

**An Antisense Approach to  
Understanding Glyoxylate Cycle  
Function in Higher Plants**

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## ABSTRACT

The glyoxylate cycle is a key step in the conversion of storage lipids to sugars in germinating oilseeds. This conversion is thought to allow seedling growth prior to the development of photosynthetic organs. The glyoxylate cycle is thought not only to operate during this stage of development but also during senescence, starvation, pollen development and embryogenesis. It is not known how important the cycle is for plant physiology and development. Two enzymes, isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MS, EC 4.1.3.2), are central to the cycle. In order to elucidate the function of the glyoxylate cycle in plant development, it was proposed that the synthesis of MS and ICL be inhibited in transgenic plants transformed with chimaeric antisense *Ms* or *Icl* genes. Two plant species each offering specific advantages, *Nicotiana plumbaginifolia* and *Solanum tuberosum*, were selected for these experiments. Partial *Ms* and *Icl* cDNA fragments from both species were isolated and cloned. These were used to construct plant transformation plasmids in which each cDNA fragment was in the antisense orientation with respect to the CaMV 35S promoter. Transgenic *N. plumbaginifolia* and *S. tuberosum* plants were regenerated from tissue transformations using strains of *Agrobacterium tumefaciens* carrying the respective plasmids. The presence of either transgene did not detectably interfere with fertility, germination or post-germinative growth in *N. plumbaginifolia*. In addition to the *Ms* and *Icl* mRNAs, the majority of *N. plumbaginifolia* transformants synthesised a transcript not detected in non-transformants, which was shown in antisense *Ms* transformants to be antisense RNA complementary to the *Ms* cDNA fragment. Despite the abundance of antisense RNA in some transformants, neither the steady-state levels of target gene mRNA, nor MS enzyme activities, were significantly affected. The relevance of these results to the mechanism of antisense inhibition is discussed. Evidence was obtained that suggests that the expression of *Ms* and *Icl* is influenced by multiple factors.



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## ABBREVIATIONS

A	adenine
A (amino acid)	alanine
A <sub>260</sub>	absorbance at 260 nanometres
A <sub>600</sub>	absorbance at 600 nanometres
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
C (amino acid)	cysteine
°C	degrees Celsius
cAMP	adenosine 3',5'-cyclic monophosphate
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
Ci	curie
CoA	coenzyme A
Da	dalton
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
ds	double stranded
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate



EDTA	ethenediaminetetraacetic acid (disodium salt)
g	gramme
<i>g</i>	relative centrifugal force
G	guanine
h	hour
H	histidine
I	isoleucine
J	joule
K	lysine
kb	kilobase
kbp	kilobase pair
l	litre
L	leucine
lb in <sup>-2</sup>	pounds per square inch
m	metre
M	molar
M (amino acid)	methionine
min	minute
μmol	micromole
<i>M<sub>r</sub></i>	relative molecular mass
mRNA	messenger RNA
NAD <sup>+</sup>	nicotinamide adenine nucleotide (oxidised form)
NADH	nicotinamide adenine nucleotide (reduced form)
NTP	nucleoside 5'-triphosphate
PCR	polymerase chain reaction
Q	glutamine
R	arginine
RNA	ribonucleic acid



RNase	ribonuclease
S	serine
SDS	sodium dodecyl sulphate
sec	second (s in expressions)
ss	single stranded
T	thymine
Ti	tumour inducing
Tris	tris-(hydroxymethyl)-methylamine
U	units
UTP	uridine 5'-triphosphate
UV	ultra violet
V	valine
(v/v)	volume:volume ratio
(w/v)	weight:volume ratio



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# **1. INTRODUCTION**



## 1.1 Aims of the introduction

The glyoxylate cycle has been extensively studied as a model system for understanding the regulation of gene expression in plants. Most of the enzyme activities associated with the glyoxylate cycle are localised in a defined cellular compartment, the peroxisome, and the glyoxylate cycle functions both at specific developmental stages and in response to changes in metabolic status. Two enzymes are unique to this cycle, malate synthase (MS, EC 4.1.3.2) and isocitrate lyase (ICL, EC 4.1.3.1).

Although many aspects of the biochemistry of the glyoxylate cycle and the regulation of glyoxylate cycle genes have been elucidated, the function and control of the cycle at specific stages of development and in relation to whole plant physiology is still poorly understood. In this introduction, current understanding of peroxisome biogenesis and organisation, the biochemistry of the cycle and associated processes, and the regulation of glyoxylate cycle gene expression will be discussed.

## 1.2 Peroxisome biogenesis and organisation

### 1.2.1 *Structure of peroxisomes*

Peroxisomes are a class of organelles present in a wide variety of eukaryotic cell types. In plants they are present in essentially all cells and vary in size from 0.1  $\mu\text{m}$  to 1.8  $\mu\text{m}$  depending on species and tissue (Huang *et al.*, 1983). All are bounded by a single outer membrane and have simple ultrastructure, lacking internal membranes and an organellar genome. Although all share certain characteristic enzymes such as  $\text{H}_2\text{O}_2$ -producing oxidases and catalase, plant peroxisomes can be divided into several categories according to their enzymatic content and hence biochemical function. Three classes, the glyoxysomes of oilseed species, leaf-type peroxisomes in photosynthetic tissue and those involved in ureide metabolism in the root nodules of leguminous plants, are discussed in section 1.3.



### 1.2.2 Peroxisome formation

Peroxisomes were thought originally to be derived by vesiculation of the endoplasmic reticulum (ER), but evidence now suggests that peroxisome biogenesis occurs through the fission of existing organelles, followed by growth involving uptake of proteins synthesised on free polyribosomes (for reviews, see Lazarow & Fujiki, 1985 and Subramani, 1993). The two processes of proliferation and growth are functionally separable. Proliferation of peroxisomes may precede growth, as in the yeast *Candida boidinii* when shifted from glucose to methanol as carbon source (Veenhuis & Goodman, 1990), or follow prolonged growth, as in the yeast *Hansenula polymorpha* in response to similar treatment (Veenhuis *et al.*, 1979). The peroxisomes of the *pas8* mutant of the yeast *Pichia pastoris* are unable to grow due to a specific deficiency in protein import, but are able to divide and segregate to daughter cells (McCollum *et al.*, 1993).

Peroxisomes do not appear to form *de novo* in cells (Lazarow & Fujiki, 1985; Subramani, 1993). *Saccharomyces cerevisiae* cells grown on glucose have been found to possess at least one small peroxisome which grows and proliferates when glucose is removed from the growth medium (Aitchison *et al.*, 1992). However, in the temperature-sensitive *per13-6<sup>ts</sup>* mutant of *H. polymorpha*, which completely lacks peroxisomes when grown at the restrictive temperature, peroxisomes appear to form *de novo* when cells are shifted from the restrictive temperature to the permissive temperature (Waterham *et al.*, 1993). This suggests that fission of pre-existing peroxisomes may not be the single universal pathway for peroxisome biogenesis.

The origin of peroxisomes in plants is poorly understood. Glyoxysomes in the lipid storage tissues of oilseeds undergo significant enlargement during germination, requiring input of membrane lipids which glyoxysomes cannot themselves synthesise (Lin *et al.*, 1982). In cotton cotyledon glyoxysomes, *in vivo* pulse-chasing experiments have demonstrated that the phospholipids of which the glyoxysomal membranes consist do not originate from the ER (Chapman & Trelease, 1991). *In*



*vitro* studies have suggested that lipid bodies, which contain the storage triacylglycerols used for pre-photosynthetic growth in oilseed species (section 1.3.1), also provide phospholipids which make up glyoxysomal membranes (Chapman & Trelease, 1991). Peroxisomes in many plant tissues appear to undergo exclusively expansion rather than division (Baker, in press). Root nodule peroxisomes with reduced levels of uricase, a major protein component of these organelles, show a proportional decrease in size (Lee *et al.*, 1993), suggesting that peroxisomal enlargement is driven at least in part by accumulation of matrix proteins.

### **1.2.3 *The single-population model for glyoxysomes and leaf peroxisomes***

Peroxisomes in the cotyledonary cells of oilseed species perform two successive and distinct metabolic roles following germination (Becker *et al.*, 1978). In germinating oilseeds, only glyoxysomes, involved in the mobilisation of storage lipid for pre-photosynthetic growth, are present. Following germination of epigeal seeds, the cotyledons become photosynthetic organs ('greening'). During this transition from heterotrophy to autotrophy, leaf peroxisomes, involved in photorespiration, appear along with chloroplasts (Kagawa & Beevers, 1975). Despite their functional differences, evidence suggests that glyoxysomes and leaf peroxisomes are derived from the same peroxisomal population which changes enzymatic content and function during plant development. This has been referred to as the 'single-population' model (Trelease *et al.*, 1971), in contrast to a scheme in which glyoxysomes and leaf peroxisomes are formed and destroyed independently.

Strong evidence in support of the one-population model has been obtained from immunocytochemical analysis of greening cotyledons. In cucumber, use of gold-labelled antibodies to glyoxysome-specific ICL and the leaf peroxisome-specific photorespiratory enzyme serine:glyoxylate aminotransferase (SGAT) has shown that these enzymes can exist in the same peroxisome during the transitional stage of greening (Titus & Becker, 1985). Similar results were obtained in pumpkin with immuno-labelling of glyoxysomal citrate synthase and leaf peroxisome-specific



glycolate oxidase (GO) (Nishimura *et al.*, 1986) and with ICL and leaf peroxisome-specific hydroxypyruvate reductase (HPR) in watermelon (Sautter, 1986). Furthermore, leaf peroxisomes can import glyoxysomal proteins, suggesting that different types of peroxisome share a common import mechanism (see section 1.2.4.4). Glyoxysome-specific MS translated *in vitro* can be imported into glyoxysomes, leaf peroxisomes and ‘intermediary’ peroxisomes isolated from greening cotyledons, but appears to be degraded specifically in intermediary microbodies only (Mori & Nishimura, 1989). This suggests that leaf peroxisomes develop from glyoxysomes and that they do so by specific degradation of the glyoxysomal components during greening. A reversal of this process occurs during leaf senescence (see section 1.4.5).

#### **1.2.4 Import of proteins into peroxisomes**

##### **1.2.4.1 The class I peroxisomal targeting signal**

Peroxisomes have no organellar genome and peroxisomal proteins are encoded by nuclear genes. In common with nuclear-encoded mitochondrial and chloroplast proteins, peroxisomal proteins are synthesised on free polyribosomes and undergo post-translational import into peroxisomes (Lazarow and Fujiki, 1985). However, unlike mitochondrial and chloroplastic protein import, most peroxisomal proteins do not undergo proteolytic processing. In plants, this includes MS (Kruse *et al.*, 1981), GO (Gerdes *et al.*, 1982) and ICL (Roberts & Lord, 1981; Maeshima *et al.*, 1988). The absence of proteolytic processing as part of peroxisomal import must mean that the targeting signal for such proteins resides in the mature polypeptide. The import of most peroxisomal proteins in this category appears to be dependent on a conserved tripeptide motif located near, or at, the C-terminus. This was first identified through studies on the firefly luciferase protein (Gould *et al.*, 1987) and is now referred to as a class I peroxisomal targeting signal (PTS1). Firefly luciferase is translocated to peroxisomes both in firefly cells and when translated in mammalian cells. Gene deletion and linker insertion identified two polypeptide segments necessary for



correct targeting of this protein (Gould *et al.*, 1987). One of these, a short segment at the extreme C-terminus, was capable of redirecting cytoplasmic mutants of luciferase to peroxisomes in addition to bacterial chloramphenicol acetyl transferase (CAT), normally cytoplasmic in mammalian cells, and murine dihydrofolate reductase, also cytoplasmic (Gould *et al.*, 1987). The analogous segments from four unrelated peroxisomal proteins were all capable of directing cytosolic proteins to peroxisomes (Gould *et al.*, 1988). Detailed mutagenesis of this segment in luciferase (Gould *et al.*, 1989) and rat acyl-CoA oxidase (AOX) (Miura *et al.*, 1992) identified a tripeptide sequence to which only a limited number of conserved amino acid changes could be made without abolishing import activity. This motif, found as S-K-L in luciferase, has a consensus sequence S/A/C-K/R/H-L.

Luciferase is peroxisomally-targeted when expressed in transgenic tobacco and yeast, and a fusion protein containing the C-terminus of a yeast peroxisomal protein, PMP20, was correctly targeted when expressed in CV-1 monkey kidney cells (Gould *et al.*, 1990). This shows that the import process is conserved, at least in part, between plants, insects, yeasts and mammals. In addition, antibodies raised to peptides ending in S-K-L recognise peroxisomal proteins in fungi, plants and animals (Keller *et al.*, 1991). The ability of the six C-terminal amino acids of spinach leaf peroxisome GO, containing a putative PTS1, to target  $\beta$ -glucuronidase (GUS) to peroxisomes in transgenic tobacco (Volokita, 1991) is direct confirmation that a sequence containing a putative PTS1 can mediate peroxisomal targeting in plants. In addition, cottonseed ICL is successfully targeted to the peroxisomes of transgenic monkey CV-1, mouse L, HeLa and CHO cells and import is dependent on the presence of the C-terminus which contains a putative PTS1 sequence (Trelease *et al.*, 1994). The 37 C-terminal amino acids of *B. napus* ICL, including a putative PTS1 at the extreme terminus, is necessary for import of this protein into peroxisomes in *Arabidopsis thaliana* and the last five amino acids are sufficient to direct CAT to peroxisomes (Olsen *et al.*, 1993). However, truncation of the C-terminus does not prevent the import of castor bean ICL into sunflower glyoxysomes *in vitro*, showing that in other systems it is dispensable (Behari & Baker, 1993).



Sequence analysis has found tripeptide motifs conforming to the S/A/C-K/R/H-L consensus in a range of peroxisomal proteins (de Hoop & Ab, 1992). However, the mere presence of such a sequence is not always sufficient to target proteins correctly, as shown by the necessity for the N-terminal sequence of luciferase (Gould *et al.*, 1987). The context of the PTS1 motif within the peptide sequence has also been shown to be important in targeting (Gould *et al.*, 1989). In addition, the PTS1 motifs of many peroxisomal proteins diverge significantly from the consensus. Putative PTS1 motifs at the C-terminus of MS appear as S-K/R-L in five higher plant species (de Hoop & Ab, 1992), whereas an A-R-M (de Hoop & Ab, 1992) or S-R-M (Reynolds & Smith, 1995a; Olsen *et al.*, 1993) motif seems favoured for ICL.

#### 1.2.4.2 *The class II peroxisomal targeting signal*

Not all peroxisomal proteins contain a PTS1. Some are synthesised with an N-terminal transit peptide which is cleaved upon import. This presequence contains the class II peroxisomal targeting sequence (PTS2), a nonapeptide with consensus sequence R/K-L/V/I-X<sub>5</sub>-H/Q-L/A (Rachubinski & Subramani, 1995). The rat peroxisomal protein 3-ketoacyl-CoA thiolase (thiolase) is synthesised as a precursor with an N-terminal presequence which is cleaved upon translocation of the protein into peroxisomes (Hijikata *et al.*, 1990; Bodnar & Rachubinski, 1990). The presequence was necessary for correct targeting of thiolase, and when fused to CAT, was sufficient to direct this protein to peroxisomes (Swinkels *et al.*, 1991). Cucumber thiolase is also synthesised with a putative N-terminal PTS2 which is cleaved upon import (Preisig-Muller & Kindl, 1993) and glyoxysomal malate dehydrogenase (gMDH) from watermelon (Gietl, 1990) and cucumber (Riezman *et al.*, 1980; Kim & Smith, 1994a) is synthesised with an N-terminal transit peptide which is cleaved upon import, but in none of these three cases have the presequences been demonstrated to be essential for import. Watermelon gMDH is imported into peroxisomes when synthesised in the yeast *H. polymorpha*, but appears to remain uncleaved in this context (van der Klei *et al.*, 1993).



#### 1.2.4.3 Other targeting signals

Not all peroxisomal proteins fall clearly into either the PTS1 or PTS2 categories. A notable example is acyl-CoA oxidase (AOX). A PTS1 sequence has been shown to be necessary and sufficient for peroxisomal import of rat liver AOX (Miyazawa *et al.*, 1989), but in AOX from the yeast *Candida tropicalis*, the C-terminal region does not contain a PTS1 and is dispensable for import. Instead, *C. tropicalis* AOX contains two non-overlapping targeting signals, one within the N-terminal region and a second located in the central portion of the polypeptide, each of which is sufficient to direct import into peroxisomes (Small *et al.*, 1988). The six C-terminal amino acids of *S. cerevisiae* Catalase A, the extremity of which is a putative PTS1, can direct reporter fusion proteins to peroxisomes but is completely dispensable for import of Catalase A itself. A second targeting signal located within the N-terminal third of the polypeptide is also sufficient for peroxisomal targeting but is context-sensitive (Kragler *et al.*, 1993). The *H. polymorpha* PER1 protein has both a putative PTS1 and a putative PTS2, but either of two polypeptides of nine or sixteen amino acids containing each of these putative signals, respectively, can direct peroxisomal targeting (Waterham *et al.*, 1994).

#### 1.2.4.4 Mechanisms of import

The mechanisms by which these targeting signals mediate import of proteins into peroxisomes is poorly understood. The import of AOX into rat liver peroxisomes (Imanaka *et al.*, 1987) and castor bean ICL into sunflower glyoxysomes (Behari & Baker, 1993) has been shown to require ATP hydrolysis. Different classes of peroxisome do not appear to systematically exclude proteins which are not normally part of their enzyme complement. Castor bean and *B. napus* ICL (Onyeocha *et al.*, 1993; Olsen *et al.*, 1993) synthesised in transgenic plants and pumpkin MS translated *in vitro* (Mori & Nishimura, 1989) can be translocated to leaf peroxisomes,



suggesting significant similarity in the import mechanisms of glyoxysomes and leaf peroxisomes. The mechanisms for PTS1- and PTS2-mediated import appear to differ, however. Genetic analysis of a range of *S. cerevisiae* peroxisomal mutants has shown that a mutant (*pas7*) defective for the import of thiolase (PTS2-mediated import) is competent for the import of other peroxisomal proteins (Van Der Leij *et al.*, 1992). Conversely, peroxisomes of the *pas8* mutant of the yeast *P. pastoris* are able to import thiolase but are defective in the import of other peroxisomal proteins, including PTS1-mediated firefly luciferase (McCollum *et al.*, 1993). The PAS8 protein is peroxisomal membrane-associated, binds specifically to S-K-L peptides and it has been proposed that it is a PTS1-receptor (McCollum *et al.*, 1993). A specific deficiency in PTS1-mediated peroxisomal import is responsible for the human peroxisomal disorder Zellweger syndrome (Walton *et al.*, 1992).

The active conformation of native thiolase is homodimeric. Correct targeting can be restored to truncated thiolase lacking the PTS2 by heterodimerisation with full-length thiolase in *S. cerevisiae*, suggesting that import of this protein takes place in dimerised form (Glover *et al.*, 1994). The *PEB1* gene of *S. cerevisiae* encodes an intraperoxisomal protein necessary for PTS2-mediated import and its own import is PTS2-mediated. The Peb1p protein binds specifically to the N-terminus of thiolase, and when the N-terminus of Peb1p was fused to a truncated version of thiolase lacking the PTS2, thiolase was correctly targeted (Zhang & Lazarow, 1996). This contradicts a model for PTS2-mediated import in *S. cerevisiae* proposed by other workers (Marzioch *et al.*, 1994) suggesting that a protein encoded by the *PAS7* gene is normally cytosolic and shuttles thiolase to the peroxisomal membrane only when thiolase is present.

### **1.2.5 The peroxisomal matrix may have structure**

Despite the absence of an internal membrane system in peroxisomes, evidence suggests that a level of sub-organellar structural organisation exists. This evidence has been obtained from latency studies on spinach leaf peroxisomes (Heupel *et al.*,



1991; Heupel & Heldt, 1994). These involve measurement of peroxisomal enzyme activities in the presence or absence of an intact membrane. This ascertains the necessity of the membrane for retaining peroxisomal enzymes and thus provides a measure of the level of structural integrity of the peroxisomal matrix. In these studies, treatment of peroxisomes with low levels of detergent removed the membrane and partially disintegrated what appeared to be a dense peroxisomal matrix. This led to a release of enzyme activity into the soluble fraction compared with intact peroxisomes. Osmotic shock, however, only removed the membrane. In this case the release of enzyme activity was much lower than when detergent was used, indicating that the latency of enzymes in peroxisomes is more a function of matrix structure than of the membrane. Osmotically-shocked peroxisomes were also capable of carrying out the normal peroxisomal conversions of glycerate from glycolate, glutamate, serine and malate, and glycine from glycolate, glutamate and serine (Heupel & Heldt, 1994). This demonstrates that a degree of functional integrity is preserved as well as structural integrity, even in the absence of the membrane.

A degree of structural organisation in the protein complement of peroxisomes, possibly as multi-enzyme complexes, would allow metabolite channeling in these organelles. Specific associations between maize ICL and catalase (Khan *et al.*, 1992), and ICL and MS (Beeckmans *et al.*, 1994) have been found *in vitro*, but whether this reflects the states of these proteins in peroxisomes is not known.

### **1.3 Biochemistry of peroxisomes**

Different classes of peroxisome can be defined in higher plants according to the distinct physiological functions they fulfil. Three classes are discussed here: glyoxysomes found in the lipid storage tissues of oilseeds, leaf peroxisomes found in



photosynthetic tissue and peroxisomes involved in ureide metabolism in the uninfected root nodule cells of legumes. A fourth class, unspecialised peroxisomes, has also been defined (Huang *et al.*, 1983).

### 1.3.1 Glyoxysomes

Glyoxysomes are found in the cotyledons or endosperm of oilseeds and the scutellum of cereals during post-germinative growth. They are involved in the conversion of storage triacylglycerols into the precursors of gluconeogenesis. This process is thought to fulfil a crucial developmental requirement as it allows the mobilisation of seed lipid reserves as a carbon source for growth prior to the development of photosynthetic organs (Beevers, 1980). It involves four steps: i) Storage triacylglycerols contained within lipid bodies are broken down by lipases to fatty acids and glycerol. ii) Fatty acids are transported to the glyoxysomes where they are converted to acetyl-Coenzyme A (acetyl-CoA) by the process of  $\beta$ -oxidation. iii) Within glyoxysomes, acetyl-CoA is converted by the glyoxylate cycle to 4-carbon acids. iv) Oxaloacetate (OAA) ultimately produced by this pathway is used as the substrate for gluconeogenesis in the cytosol.

#### 1.3.1.1 Breakdown of lipids

During the early stages of seedling growth, the activity of lipases increases and leads to the hydrolysis of storage triacylglycerols into fatty acids and glycerol. Oilseed species fall into two general categories with respect to their lipase activities (Huang *et al.*, 1983). *Brassica napus*, mustard and cottonseed show acid or neutral lipase activity which is lipid body-associated. Other plants, including cucumber, soybean and peanut, appear to have glyoxysomal alkaline lipases. An alkaline lipase from castor bean glyoxysomes is membrane-bound (Maeshima *et al.*, 1987). Electron microscopy has shown proliferation and clustering of glyoxysome-like microbodies around lipid bodies in pollen of *Ophrys lutea*, concomitant with a decrease in lipid



body volume (Pais & Feijo, 1987). Lipase activity was detected in both the membrane and matrix of these organelles. Similar results have been obtained for *B. napus* pollen (Charzynska *et al.*, 1989).

#### 1.3.1.2 $\beta$ -oxidation

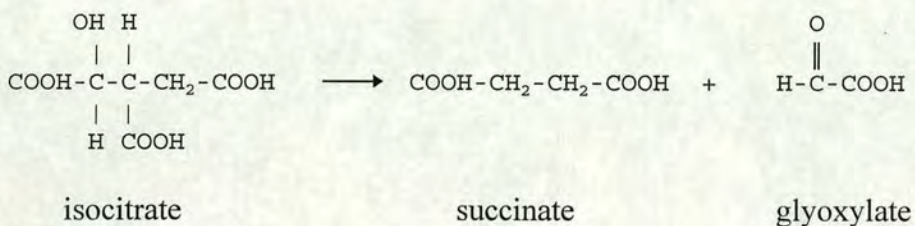
Peroxisomal  $\beta$ -oxidation was first described in glyoxysomes from germinating castor bean endosperm (Cooper & Beevers, 1969a). It consists of the following steps (van den Bosch *et al.*, 1992): fatty acids are activated to their acyl-CoA derivatives by the action of fatty acyl-CoA synthase, on the outer face of glyoxysomes. The acyl-CoA derivatives are transported across the glyoxysomal membrane and converted to enoyl-CoA derivatives by the action of acyl-CoA oxidase. This generates  $H_2O_2$ , which is degraded by catalase. The enoyl-CoA derivatives are converted to their 3-keto esters through the activities of multi-functional proteins. A multi-functional protein carries out these steps in cucumber cotyledon glyoxysomes. There are three isoforms of this protein in glyoxysomes and these differ from isoforms found in green leaves (Gühnemann-Schäfer & Kindl, 1995). Acetyl-CoA is generated from the 3-keto esters by reaction with CoA, a process catalysed by 3-oxoacyl-CoA thiolase. Whilst acetyl-CoA subsequently enters the glyoxylate cycle, these reactions result in the formation of a fatty acyl-CoA molecule which is two carbons shorter than the original molecule.

#### 1.3.1.3 The glyoxylate cycle

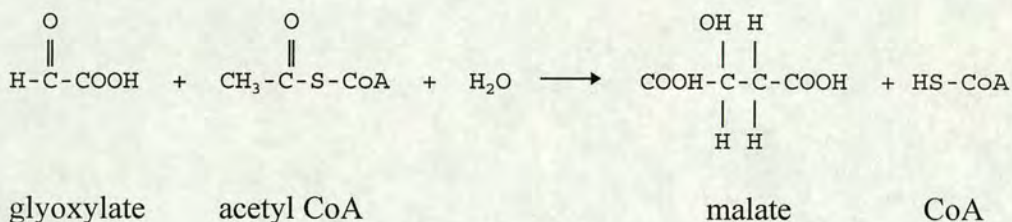
The glyoxylate cycle operates in a wide range of microorganisms and was first identified through the ability of the bacterium *Pseudomonas* to grow using acetate as sole carbon source (Kornberg & Madsen, 1957). It was subsequently found to operate in the endosperm of castor bean seedlings (Kornberg & Beevers, 1957). The initial steps of the glyoxylate cycle are the same as those of the citric acid cycle. OAA and acetyl-CoA undergo condensation to form citrate through the action of



citrate synthase. Citrate is then converted to isocitrate by the enzyme aconitase. However, the subsequent oxidative decarboxylation steps of the citric acid cycle are avoided by the action of two enzymes exclusive to the glyoxylate cycle, ICL and MS. ICL cleaves isocitrate into succinate and glyoxylate, as follows:

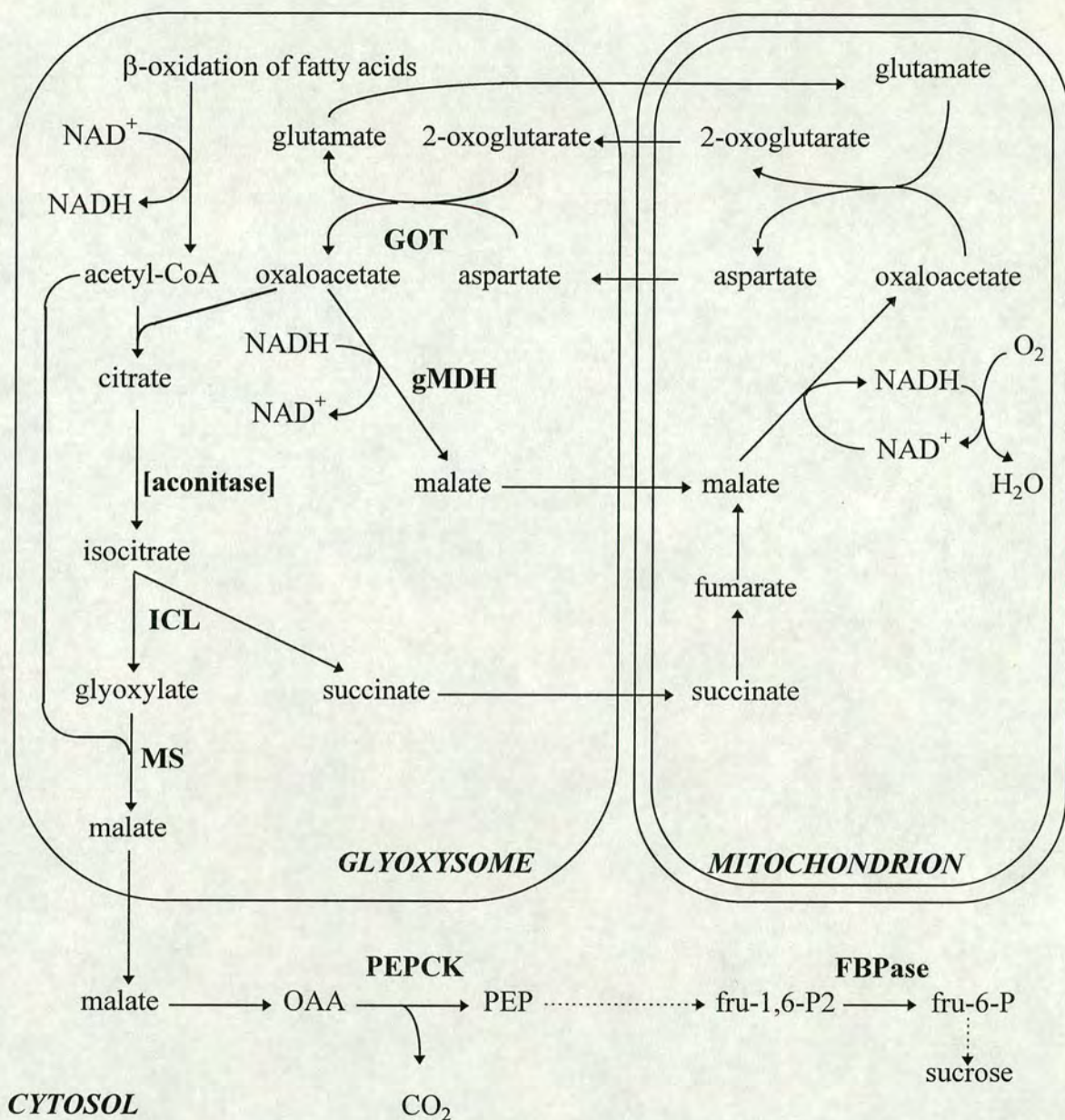


MS then catalyses the condensation of glyoxylate with a second molecule of acetyl-CoA to form malate, as follows:



Exactly how OAA for gluconeogenesis is derived from the glyoxylate cycle, whether from succinate produced by ICL or from malate produced by MS, depends on the details of the subsequent steps of the cycle which have yet to be fully elucidated. The presence of malate dehydrogenase in glyoxysomes (gMDH, Cooper & Beevers, 1969b) suggests the possibility that OAA is regenerated from malate (a cyclic scheme), with succinate acting as the substrate for gluconeogenesis. However, evidence favours an alternative model in which shuttling of reducing equivalents between glyoxysomes and mitochondria allows malate to be used as the precursor for gluconeogenesis in the cytosol (Mettler & Beevers, 1980). This model is illustrated in figure 1.1. In this scheme, gMDH acts to reduce OAA and generate malate (gMDH would catalyse the opposite reaction in the cyclic scheme). This malate is exported to the mitochondria, where it acts to generate aspartate, which is





**Figure 1.1: The malate-aspartate shuttle model of the glyoxylate cycle**

Adapted from Mettler & Beevers, 1980. **MS**: malate synthase. **ICL**: isocitrate lyase. **gMDH**: (glyoxysomal) malate dehydrogenase. **GOT**: glutamate:oxaloacetate aminotransferase. **PEPCK**: phosphoenolpyruvate carboxykinase. **FBPase**: fructose-1,6-bisphosphatase. **Aconitase** appears in brackets to indicate uncertainty of subcellular location. **OAA**: oxaloacetate. **PEP**: phosphoenolpyruvate. **Fru-1,6-P2**: fructose-1,6-bisphosphate. **Fru-6-P**: fructose-6-phosphate. Dotted lines indicate multiple steps.



shuttled back to the glyoxysomes. Here, glutamate:oxaloacetate aminotransferase (GOT) converts aspartate to OAA, which is used in two reactions: the generation of citrate (and hence malate), and the direct generation of malate by gMDH for the mitochondrial shuttle, completing the circuit. In this scheme, malate produced by MS is the precursor for gluconeogenesis.

Several lines of evidence support this model rather than the simple cyclic scheme. First, it circumvents the necessity for gMDH to regenerate OAA from malate, a highly unfavourable reaction especially in the presence of NADH. It also provides a means for maintaining adequate levels of OAA for citrate synthase activity in an organelle which produces NADH (from  $\beta$ -oxidation of fatty acids) but has no mechanism for oxidising it to  $\text{NAD}^+$  (Cooper & Beevers, 1969a). *In vitro* experiments have shown that GOT can be coupled to gMDH to oxidise NADH in purified glyoxysomes. Addition of aspartate and 2-oxoglutarate (see fig. 1.1) causes rapid oxidation of NADH accumulated in isolated glyoxysomes and this can be prevented by the addition of an inhibitor of GOT (Mettler & Beevers, 1980). Radioactive tracing of added aspartate shows conversion to malate and then sugars (Stewart & Beevers, 1967). However, transfer of the putative shuttling components in this model has not been demonstrated. Reduction and oxidation (redox) activities of glyoxysome membranes have been shown to increase concomitantly with development of glyoxylate cycle enzyme activities during castor bean germination (Alani *et al.*, 1990) and an electron transport chain has been shown to operate in glyoxysomal membranes (Fang *et al.*, 1987). This suggests the possibility that NADH can be recycled continuously within the organelle through a membrane redox protein system rather than a molecular shuttle of reducing equivalents.

Four of the five enzymes central to any representation of the glyoxylate cycle, citrate synthase, ICL, MS and gMDH are clearly present in glyoxysomes (Cooper & Beevers, 1969b). Evidence suggests that aconitase, however, is absent from glyoxysomes (Courtois-Verniquet & Douce, 1993; Hayashi *et al.*, 1995; De Bellis *et al.*, 1993, 1994) and that a cytosolic isoform may participate in the glyoxylate cycle in etiolated pumpkin cotyledons (Hayashi *et al.*, 1995).



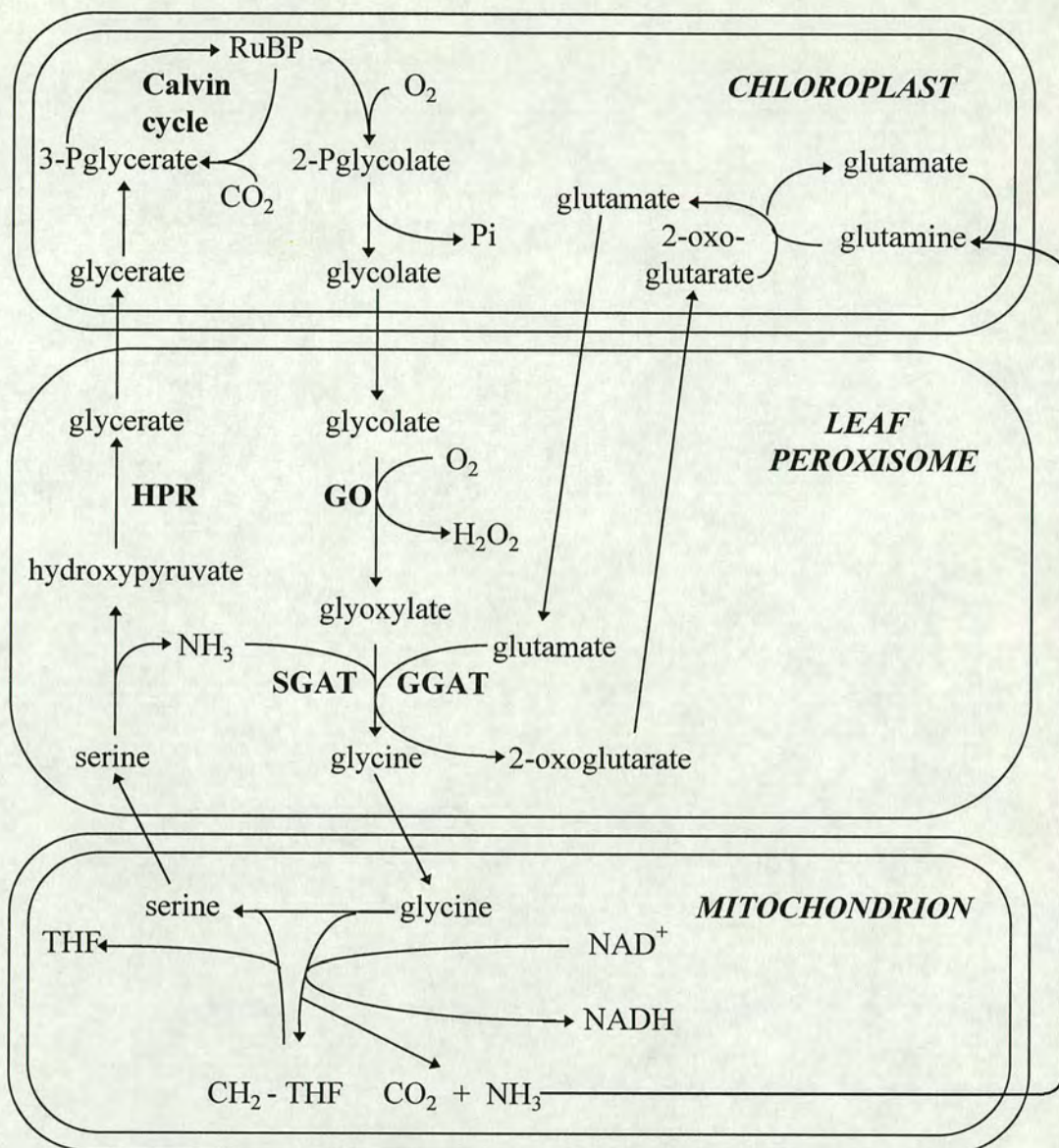
#### 1.3.1.4 Gluconeogenesis

OAA produced from malate (or succinate) is converted to sucrose by the process of gluconeogenesis. In contrast to  $\beta$ -oxidation and the glyoxylate cycle, this takes place in the cytosol in marrow cotyledons (ap Rees *et al.*, 1975; Leegood & ap Rees, 1978a) and castor bean endosperm (Nishimura & Beevers, 1979). Phosphoenolpyruvate (PEP) is produced from OAA by Phosphoenolpyruvate carboxykinase (PEPCK) and converted to triose-phosphates by a reversal of glycolysis. Fructose-1,6-bisphosphate resulting from this process is converted to fructose-6-phosphate (hexose-phosphate) by fructose-1,6-bisphosphatase (FBPase) and fructose-6-phosphate is finally converted to sucrose. The reactions catalysed by both PEPCK and FBPase are thought to be important control points for gluconeogenic flux (Leegood & ap Rees, 1978b) and this has been shown directly for PEPCK (Trevanion *et al.*, 1995). The cucumber *Pck* gene, which encodes PEPCK, is expressed during post-germinative growth and senescence (Kim & Smith, 1994b), as are *Ms* and *Icl* (see section 1.4) which supports the role of the glyoxylate cycle as providing the precursors for gluconeogenesis during these developmental stages. However, starvation treatments which induce expression of *Ms* and *Icl* (section 1.5.2.3) fail to induce expression of *Pck*, demonstrating that these genes are not coordinately regulated in all circumstances.

#### 1.3.2 Leaf peroxisomes

Leaf peroxisomes are involved in photorespiration and contain most of the enzymes of the glycolate pathway (for review, see Leegood *et al.*, 1995). Photorespiration is a process which occurs in conditions of high oxygen concentration and is initiated by the bifunctional enzyme ribulose 1,5-bisphosphate carboxylase / oxygenase (RuBisCO). This enzyme normally catalyses the carboxylation of ribulose 1,5-bisphosphate (RuBP) whereby two molecules of glycerate 3-phosphate are generated for every molecule of RuBP carboxylated. This subsequently allows the Calvin cycle





**Figure 1.2: The photorespiratory pathway and its subcellular location**

**GO:** glycolate oxidase. **SGAT:** serine:glyoxylate aminotransferase. **GGAT:** glutamate:glyoxylate aminotransferase. **HPR:** hydroxypyruvate reductase. **RuBP:** ribulose-1,5-bisphosphate. **3-Pglycerate:** glycerate 3-phosphate. **2-Pglycolate:** glycolate 2-phosphate. **THF:** tetrahydrofolic acid.



to proceed. During photorespiration however, RuBisCO oxygenates RuBP to form glycerate 3-phosphate and glycolate 2-phosphate. Glycolate 2-phosphate cannot be utilised in the Calvin cycle and would represent a functional loss of assimilated carbon if not further metabolised. The photosynthetic carbon oxidation cycle (PCO or glycolate cycle) partially recovers this carbon. The PCO involves the coordinated activities of enzymes in the chloroplast, peroxisome and mitochondrion (refer to figure 1.2). Glycolate 2-phosphate is converted to glycolate in the chloroplast and oxidised by glycolate oxidase (GO) in the peroxisome to form glyoxylate and  $H_2O_2$ .  $H_2O_2$  is converted to water and oxygen by the action of catalase. The glyoxylate produced is transaminated by either serine:glyoxylate aminotransferase (SGAT) or glutamate:glyoxylate aminotransferase (GGAT) to form glycine, which is transported to the mitochondria where the net conversion of two molecules of glycine to one molecule of serine and one molecule of  $CO_2$  takes place. Serine is returned to the peroxisome where it acts as the amino donor to glyoxylate in the transamination catalysed by SGAT, and results in the generation of hydroxypyruvate which is reduced by hydroxypyruvate reductase (HPR) to form glycerate. This is transported to the chloroplast where it is converted to glycerate 3-phosphate, which can be processed by the Calvin cycle. The PCO recovers three of the four carbon atoms from two molecules of glycolate 2-phosphate, the fourth of which is lost as  $CO_2$  (Ogren, 1984).

Leaf peroxisomes therefore contain GO, catalase, SGAT, GGAT and HPR. The amounts of HPR (Hondred *et al.*, 1987; Greenler *et al.*, 1989), SGAT (Hondred *et al.*, 1987) and GO (Ludt & Kindl, 1990) rise during greening of cotyledons from low or undetectable levels to become major peroxisomal components. Accumulation of these enzymes is regulated primarily at the transcriptional level and is light-dependent (Hondred *et al.*, 1987; Greenler & Becker, 1990).



### 1.3.3 Root nodule peroxisomes

Several genera within the Leguminosae, including species such as soybean and cowpea, are able to fix atmospheric nitrogen due to a symbiotic relationship with specific members of the bacterial genus *Rhizobium* (Rawsthorne *et al.*, 1980). This symbiotic interaction leads to the formation of nodules on the roots of the host plant which consist of infected and uninfected cells in approximately equal proportions. Whilst infected cells are involved in nitrogen fixation, uninfected cells are involved in combining fixed nitrogen with carbon to form mobilisable nitrogen-containing compounds. In soybean and cowpea, these are predominantly the ureides, allantoin and allantoic acid (Hanks *et al.*, 1981). Proliferation of peroxisomes in these cells is associated with the synthesis of nodule-specific uricase, a peroxisomal  $H_2O_2$ -generating enzyme which catalyses the synthesis of allantoin from uric acid (Nguyen *et al.*, 1985). Expression of an antisense nodulin-35 gene (the nodulin-35 gene encodes nodule-specific uricase) in transgenic *Vigna aconitifolia* root nodules interferes with the development of nodule peroxisomes and results in regenerated plants showing symptoms of nitrogen deficiency (Lee *et al.*, 1993).

### 1.4 Synthesis of MS and ICL during plant development

The enzymes ICL and MS are exclusive to the glyoxylate cycle and have been studied extensively as marker enzymes for this cycle during plant development. Their activities have been detected in higher plants during embryogenesis and post-germinative growth of several species (Allen *et al.*, 1988; Comai *et al.*, 1989; Turley & Trelease, 1990; Weir *et al.*, 1980; Zhang *et al.*, 1993), senescence and starvation (Graham *et al.*, 1992; Pistelli *et al.*, 1991; De Bellis *et al.*, 1991) and pollen formation (Zhang *et al.*, 1994).



#### 1.4.1 *The Ms gene and protein*

In cucumber, the *Ms* gene appears to exist as a single copy (Graham *et al.*, 1989). In *B. napus*, at least four different classes of gene comprise an *Ms* gene family, each member of which is present in at least one copy per haploid *B. napus* genome. However, of these, only a single class of *Ms* gene appears to be expressed during germination (Comai *et al.*, 1992).

The cucumber *Ms* gene (Graham *et al.*, 1989) has a transcribed sequence of 2582 nucleotides which consists of four exons of 393, 327, 330 and 654 nucleotides (in order from the 5' end of the gene to the 3' end) and three introns of 421, 384 and 73 nucleotides between these, respectively. The mature mRNA is approximately 1900 nucleotides in length. The complete reading frame of 1704 nucleotides encodes a protein of 568 amino acids. This has a predicted size of 64961 Da. The predicted size of this protein is in agreement with the experimentally determined size of the MS protein in cucumber, at approximately 63000 Da (Becker *et al.*, 1978).

A putative peroxisomal targeting sequence S-K-L (PTS1) is present at its C-terminus. The MS protein does not undergo detectable proteolytic processing and does not appear to be a glycoprotein (Kruse & Kindl, 1983). In its native glyoxysomal state, MS is thought to exist as an octamer (Kruse & Kindl, 1983).

#### 1.4.2 *The Icl gene and protein*

*B. napus* appears to have six *Icl* genes per genome which can be classified into two subfamilies according to 5' and 3' untranslated sequence. Evidence suggests that members of both classes are expressed during development (Zhang *et al.*, 1993). Cucumber appears to have a single *Icl* gene (Reynolds & Smith, 1995a).

The transcribed sequence of the cucumber *Icl* gene is 2369 nucleotides in length and contains three exons of 30, 1603 and 95 nucleotides (5' to 3') respectively, and two small introns of 87 and 89 nucleotides between these,



respectively (Reynolds & Smith, 1995a). In contrast, a *B. napus Icl* gene contains four introns (Zhang *et al.*, 1993). The mature cucumber *Icl* mRNA is approximately 2100 nucleotides long. The complete reading frame of 1728 nucleotides encodes a protein of 576 amino acids. This has a predicted size of 64618 Da (Reynolds & Smith, 1995a). The *Icl* coding sequences identified from cotton (Turley *et al.*, 1990), castor bean (Beeching & Northcote, 1987) and *B. napus* (Comai *et al.*, 1992) cDNAs are all approximately 1700 nucleotides in length and estimates of the size of the ICL polypeptide monomer in species other than cucumber are all approximately 64000 Da, though estimates vary from about 62000 Da in castor bean (Roberts & Lord, 1981) to 64000 Da in maize (Khan *et al.*, 1992) and 64741 Da in cotton (Turley *et al.*, 1990).

The C-terminal S-R-M tripeptide of cucumber (Reynolds & Smith, 1995a) and *B. napus* (Olsen *et al.*, 1993) ICL is a putative PTS1 sequence which appears as A-R-M in cotton (Turley *et al.*, 1990) and castor bean (Beeching & Northcote, 1987). The ICL protein does not undergo detectable proteolytic processing (Roberts & Lord, 1981) and does not appear to be a glycoprotein (Riezman *et al.*, 1980; Khan *et al.*, 1992). It appears to exist as a tetramer in its native state (Khan *et al.*, 1992). A segment of 52 amino acids in the peptide sequences from plants appears to be absent from the sequence of the *E. coli icl* sequence (Reynolds & Smith, 1995a).

#### **1.4.3 Synthesis of MS and ICL during post-germinative growth**

Post-germinative growth of oilseeds is dependent on storage lipids as a carbon source until photosynthetic organs are produced. As a central part of the conversion of triacylglycerols into carbohydrate, the glyoxylate cycle must be active during this phase of development. In plants, it was first demonstrated to operate at this stage in castor bean endosperm (Kornberg & Beevers, 1957). During early post-germinative growth in cucumber, ICL and MS enzyme activities rise, in parallel, from undetectable levels in dry seed, to a peak at four days after seed imbibition, then fall



to undetectable levels by day seven (Becker *et al.*, 1978). Greening of cotyledons was complete by day five.

These changes took place against a background of lipid breakdown and followed increases and subsequent decreases in translatable mRNA for these proteins (Weir *et al.*, 1980). Northern hybridisation experiments using a cucumber *Ms* cDNA probe have subsequently shown that these changes are attributable to increases in *Ms* mRNA and not simply a function of mRNA translatability (Smith & Leaver, 1986). A genomic fragment containing the entire cucumber *Ms* coding region with 1856 base pairs of sequence upstream of the transcriptional start site was transferred into petunia and *Nicotiana plumbaginifolia* (Graham *et al.*, 1990). This resulted in the same peak of expression of this gene following germination as in its endogenous context. A fragment containing these putative promoter sequences was fused to the *gus* reporter gene and transferred into *N. plumbaginifolia*. GUS activity was measured and showed a peak similar to that observed for MS activity in cotyledons during germination. This showed that proximal upstream *cis*-acting sequences of the cucumber *Ms* gene contain sufficient information to direct faithful expression of the gene, and further supported the view that regulation of *Ms* mRNA levels in cotyledons during germination is chiefly a result of transcriptional control, as post-transcriptional control processes such as mRNA processing or degradation would be unlikely to operate for the *gus* transcript in the same way as *Ms*.

Thus, in cucumber, the increase in MS enzyme activity appears to be a result of an increase in transcription of the *Ms* gene, and the decline of MS activity follows a decrease in the levels of its mRNA. Similar patterns have been found in other plant species for *Ms* and *Icl*. Sunflower *Icl* mRNA accumulates and declines twenty four hours before a rise and fall in immunologically detectable ICL protein levels (Allen *et al.*, 1988). The mRNAs for both *Ms* and *Icl* reach peaks in a coordinate manner twenty four hours before their corresponding enzyme peaks in cotton seedlings (Turley & Trelease, 1990) and similar accumulations of *Ms* and *Icl* mRNA occur during germination of *B. napus* (Comai *et al.*, 1989). In this case, nuclear run-off experiments suggested that increase in transcriptional activity of the *Ms* and *Icl* genes



was primarily, but not solely, responsible for the increase in their mRNA levels. Although MS and ICL activities in seedlings were found to be approximately the same in *B. napus*, the quantitative ratio of the MS protein to its mRNA was significantly higher than that for ICL (Ettinger & Harada, 1990). This suggests that translational or post-translational processes may also influence the regulation of MS and ICL activities in *B. napus*. Post-transcriptional regulation of glyoxylate cycle gene expression is known to exist in some microorganisms. In *S. cerevisiae*, addition of glucose to the growth medium causes phosphorylation of ICL, resulting in a significant decrease in activity (López-Boado *et al.*, 1988). This suggests ICL is active when in a dephosphorylated state and is inactivated by phosphorylation. Castor bean MS (Yang *et al.*, 1988) and cucumber ICL (Finnessy *et al.*, 1994) have been found to be subject to phosphorylation by a glyoxysomal protein kinase, but the significance of this is not known.

#### **1.4.4 Synthesis of MS and ICL during embryogenesis**

Enzymes involved in  $\beta$ -oxidation, the glyoxylate cycle and gluconeogenesis can be detected during embryogenesis (Miernyk & Trelease, 1981; Bortman *et al.*, 1981; Choinski & Trelease, 1978; Turley & Trelease, 1990). The activities of MS and ICL can be detected as early as 17 days post anthesis (DPA) in cotton, but the activities, protein and transcript levels of the two enzymes are not clearly coordinated as they are during post-germinative growth (Turley & Trelease, 1990; Choinski & Trelease, 1978). During late embryogenesis in *B. napus*, however, *Ms* and *Icl* transcripts appear to accumulate in coordination, from approximately 37 DPA (Comai *et al.*, 1989). In sunflower, *Icl* mRNA, but not protein, is detectable in embryos 19 days after flowering (Allen *et al.*, 1988).

Although more than one class of *Icl* and *Ms* gene is expressed in *B. napus* during embryogenesis and post-germinative growth, accumulation of ICL and MS during each of these stages is not due to alternate expression of distinct *Icl* or *Ms* genes (Zhang *et al.*, 1993; Comai *et al.*, 1992). However, a specific *B. napus* *Ms* gene



which is expressed during both embryogenesis and post-germinative growth is subject to differing spatial regulation in the developing vascular bundle during these stages (Comai *et al.*, 1992). Deletion analyses of the *B. napus Icl* promoter directing *gus* expression have indicated that distinct parts of the promoter are chiefly responsible for gene expression during embryogenesis and post-germinative growth (Zhang *et al.*, 1996).

#### 1.4.5 *Synthesis of MS and ICL during senescence*

Leaf senescence is a distinct developmental phase characterised by loss of cellular structure and function. It is thought to be a genetically programmed event rather than a process of passive deterioration (Steward & Bidwell, 1991). Cellular components are broken down in an ordered pattern, and breakdown products are metabolised and redirected to other parts of the plant whilst the leaf dies. Disassembly of different cellular membranes during senescence does not occur simultaneously. Chloroplast thylakoids are broken down early in senescence, stromal before granal, whilst the chloroplast envelope retains its integrity until late in senescence. Outer and vacuolar membranes participate in what is believed to be an autocatalytic process of phospholipid degradation (Paliyath & Droillard, 1992).

MS and ICL have been found to undergo a phase of activity during senescence. This was first identified as a large increase of MS and ICL activities in barley leaves immediately following detachment from the plant and dark-incubation (Gut & Matile, 1988), a treatment assumed in such studies to artificially simulate natural senescence. Levels prior to detachment were undetectable but activity reached a peak at day six of treatment. Similar peaks have been shown to occur for MS and ICL activities in leaf-beet, rice, wheat (De Bellis *et al.*, 1990) and spinach leaves (Landolt & Matile, 1990) under the same or similar conditions. These activities rose to levels similar to those detected during germination. In cucumber, rises in MS transcripts and protein levels are induced in cotyledons, leaves and roots



by excision and dark-treatment (Graham *et al.*, 1992; McLaughlin & Smith, 1994) and this is also the case for ICL in cotyledons (McLaughlin & Smith, 1994).

Excision and dark-incubation of leaves is unlikely to accurately simulate the processes of natural senescence as this treatment triggers transcription of a large number of genes not transcribed during natural senescence (Becker & Apel, 1993). However, rises in MS and ICL activity are not a response exclusive to such treatment. Similar peaks occur in naturally senescing leaves of wheat and rice (Pistelli *et al.*, 1991). In naturally senescing cucumber cotyledons, *Ms* and *Icl* transcripts (Kim & Smith, 1994a,b) and immunologically detectable protein levels (McLaughlin & Smith, 1995) show similar patterns, as do activities and immunologically detectable levels of MS and ICL in detached and naturally senescing pumpkin cotyledons (De Bellis & Nishimura, 1991).

In cucumber, *Ms* transcripts appear in naturally senescing cotyledons, leaves and petals (Graham *et al.*, 1992). Cotyledon *Ms* mRNA accumulations at germination and senescence are directly reflected by the appearance of MS protein. Detachment and dark-incubation of leaves of transgenic *N. plumbaginifolia* containing an MS promoter fragment linked to the *gus* reporter gene (Graham *et al.*, 1992) resulted in GUS expression which reflected the rise of *Ms* mRNA in detached cucumber leaves. This shows that a decrease in mRNA turnover is unlikely to account for the accumulation of *Ms* mRNA in response to this treatment. As with germination, therefore, the peaks in MS and ICL activity during senescence appear likely to be controlled primarily at the transcriptional level.

Excision of leaves is not necessary for induction of MS and ICL activity. Transfer of complete plants from light to darkness also triggers this response (Birkhan & Kindl, 1990; Graham *et al.*, 1992; Pistelli *et al.*, 1991). Additionally, levels of MS (Weir *et al.*, 1980; Smith & Leaver, 1986) and ICL (Weir *et al.*, 1980; Allen *et al.*, 1988) mRNA and protein persist in seedlings germinated and grown in darkness well after levels have declined in light-grown seedlings.  $\beta$ -oxidation activity concomitant with MS and ICL activity has been shown in some cases of excision and dark-treatment (De Bellis *et al.*, 1990). Galactolipids, a major constituent of



thylakoid lipids, undergo breakdown during leaf senescence. Experiments to follow the metabolism of radioactively-labelled galactolipids in senescent barley leaves showed partitioning of radioactivity in sugars (Wanner *et al.*, 1991). This suggests that gluconeogenesis, from precursors provided by  $\beta$ -oxidation and the glyoxylate cycle, may be occurring during senescence. Thylakoid lipids could provide the initial precursors for this pathway. The increase in MS and ICL activities following excision and dark-incubation of barley leaves (Gut & Matile, 1988) was correlated with a decline in monogalactosyl diacylglycerol, a thylakoid lipid, but such a correlation was not found in similar experiments with cucumber cotyledons (McLaughlin & Smith, 1994). This discrepancy makes it unlikely that the products of lipid breakdown act as the signal for induction of *Ms* and *Icl* gene expression. Evidence now provides support for the proposal (Graham *et al.*, 1992) that synthesis of these enzymes is repressed by high levels of sucrose and induced following the fall of sucrose levels in artificially or naturally senescent tissue. This is discussed in section 1.5.2.

Co-sedimentation of enzyme activities characteristic of both peroxisomes and glyoxysomes has been shown in senescing petals and cotyledons of pumpkin (De Bellis *et al.*, 1991) and spinach leaves (Landolt & Matile, 1990). This suggests that a direct transition from peroxisomes to glyoxysomes occurs during senescence. Immunolabelling experiments have shown this to be a reversal of the single-population glyoxysome to peroxisome transition which occurs during post-germinative growth (Nishimura *et al.*, 1993). The presence of enzymes involved in three distinct peroxisomal functions (glyoxysomal enzymes, GO and uricase) in the peroxisomes of senescent tissue have led some workers (Vicentini & Matile, 1993) to refer to these organelles as gerontosomes.

#### **1.4.6 Synthesis during pollen formation**

Proliferation and clustering of peroxisome-like organelles around lipid bodies in developing pollen of the orchid *Ophrys lutea*, concomitant with a decrease in lipid



body volume and a simultaneous increase in volume of the peroxisome-like organelles, has been shown in ultrastructural studies (Pais & Feijo, 1987). Lipase activity in the membrane and matrix of these organelles was detected. Similar results have been obtained for *B. napus* pollen (Charzynska *et al.*, 1989). This suggests that these organelles are involved in lipid breakdown. The *Ms* and *Icl* genes have also been shown to be expressed in developing pollen of *B. napus* (Zhang *et al.*, 1994). Together, these data suggest strongly that the glyoxylate cycle is involved in pollen development.

## **1.5 Metabolic regulation of *Ms* and *Icl* gene expression**

### **1.5.1 *Carbohydrate control of gene expression in bacteria and lower eukaryotes***

The expression of many genes in microorganisms is repressed in response to glucose. This is thought to be an energy-saving response, as it primarily applies to genes involved in metabolising other carbon sources and are therefore dispensable in the presence of glucose (Ronne, 1995). In bacteria, this phenomenon is termed carbon catabolite repression. In *E. coli*, the genes of the *ace* operon which encode MS and ICL are subject to carbon catabolite repression. They are required for growth of *E. coli* when acetate or fatty acids are the sole carbon source (Maloy & Nunn, 1982). Control of the *ace* operon is exerted at the transcriptional level by two repressor proteins, the *iclR* and *fadR* gene products, both of which are required for full repression of the *ace* operon (Maloy & Nunn, 1982). The *fadR* gene is also involved in the regulation of the *fad* regulon which is induced during growth on long chain fatty acids. The IclR protein recognises a 35 bp palindromic sequence in the operator/promoter region of the *ace* operon which overlaps the -35 recognition site for RNA polymerase. This interaction is impaired by PEP, but is insensitive to acetate, acetyl-CoA, pyruvate and OAA (Cortay *et al.*, 1991). Glucose is thought to mediate carbon catabolite repression in *E. coli* by causing a reduction in the levels of



cAMP. This results in inactivation of the CAP protein which is itself an activator of catabolite-repressed genes (Saier, 1989).

In *S. cerevisiae*, genes subject to glucose repression include the *ICL1* and *MLS1* genes (which encode ICL and MS, respectively), gluconeogenic genes (*PCK1*, which encodes PEPCCK and *FBP1*, which encodes FBPase) and genes encoding proteins which take-up and metabolise other carbon sources, including the *GAL*, *SUC* and *MAL* genes (Schöler & Schüller, 1994; Ronne, 1995). Repression of *ICL1* is primarily at the transcriptional level (Schöler & Schüller, 1993). A protein kinase, Snf1, has been found to be required for expression of all glucose repressed genes and cells lacking the *SNF1* gene are unable to use carbon sources other than glucose (Celenza & Carlson, 1986; Schüller & Entian, 1987). Glucose repression of genes involved in gluconeogenesis appears to involve more than one mechanism. A DNA-binding zinc finger protein encoded by the *MIG1* gene is thought to act directly upon repressible genes. Disruption of *MIG1* in cells that lack Snf1 restores expression of the *ICL1* gene, but only partially, suggesting other repressing mechanisms are acting in parallel (Schöler & Schüller, 1994).

The *FBP1* promoter contains binding sites for Mig1 and an activator protein, the binding of which is inhibited by glucose (Niederacher *et al.*, 1992). Loss of *MIG1* alone does not allow expression of *FBP1* in the presence of glucose (Lundin *et al.*, 1994). It has been proposed that control of *FBP1* involves both inhibition of the activator complex and direct repression by Mig1 (Niederacher *et al.*, 1992). Similar Mig1-binding and Snf1-dependent activator complex-binding motifs are present in the promoters of the *ICL1* gene and other gluconeogenesis-related genes (Schöler & Schüller, 1993, 1994). Though the trigger for glucose repression in *S. cerevisiae* has not been elucidated, hexokinase is thought to play an important role. Mutants in the major hexokinase *HXK2* gene do not display glucose repression. The enzymatic activity of hexokinase appears to be required for repression (Ma *et al.*, 1989).

In *Aspergillus nidulans*, ICL and MS activity is induced by acetate (Armstrong *et al.*, 1976) and ICL activity is subject to repression by sucrose (Kelly & Hynes, 1977). The *facB* gene is necessary for acetate-dependent induction of ICL and MS (Armstrong *et al.*



*al.*, 1976) and is thought to encode a *trans*-acting regulatory protein (Katz & Hynes, 1989). The *A. nidulans creA* gene is a homologue of *MIG1* (Dowzer & Kelly, 1991) and the promoter of the *acuD* gene (encoding ICL) contains binding sites for both the *facB* and *creA* gene products (De Lucas *et al.*, 1994).

In *Chlorella fusca*, synthesis of ICL is repressed if cells are allowed to photosynthesise or are provided with glucose. Glucose repression occurs even in the presence of acetate, which induces synthesis of ICL when cells are incubated in darkness (McCullough & John, 1972). Transcription of the *Ms* gene in *Euglena* is induced by ethanol and acetate. Induction is more pronounced in darkness (Woodcock & Merret, 1980).

### **1.5.2 Carbohydrate control of gene expression in higher plants**

The expression of a variety of genes in higher plants is either induced or repressed in response to the availability of sugars. By sensing metabolite levels, it is thought that plants are able to control the production of assimilates in source tissues, such as mature leaves and storage tissues during post-germinative growth, and allocation (partitioning) of these assimilates to specific sink tissues. These include storage sink tissues, such as developing seeds and tubers, or metabolic sinks which require but do not accumulate assimilates, such as meristems and roots (Sonnewald & Willmitzer, 1992). The status of a particular tissue or organ with respect to the import or export of assimilates is not static. In potato, at least four transitions from sink to source occur: during the development of etiolated sprouts to green stems, during the maturation of leaves, and during the development of seeds and tubers (Sonnewald *et al.*, 1994). In addition to changes solely at the metabolic level, alteration of correct partitioning can lead to gross morphological changes. For example, transgenic plants with reduced ADP-glucose pyrophosphorylase (AGPase), which is involved in starch biosynthesis, display significantly altered tuber formation (Müller-Röber *et al.*, 1992).



### 1.5.2.1 Induction of gene expression

The expression of several genes involved in tuber formation in potato is induced by elevation of sucrose levels, a condition which would be expected to stimulate the accumulation of starch in tubers. The expression of the *AgpaseS* gene, which encodes one of two AGPase subunits (involved in starch biosynthesis), is strongly induced by sucrose and other metabolisable carbohydrates (Müller-Röber *et al.*, 1990). Steady-state levels of sucrose synthase mRNA in potato leaves and petioles, where the sucrose synthase gene is normally inactive, increase when single-leaf stem cuttings are incubated in sucrose (Salanoubat & Belliard, 1989).

In maize, two genes both encoding sucrose synthase, *Sh1* and *Sus1*, respond in different ways to sucrose levels. Expression of *Sus1* is induced by an increase in sucrose levels (Koch *et al.*, 1992), whilst the *Sh1* gene is repressed (Maas *et al.*, 1990; Koch *et al.*, 1992). However, the expression of these two genes is localised to different tissues and this is thought to provide a mechanism for altering the distribution of sucrose synthase activity in response to changing carbohydrate status (Koch *et al.*, 1992).

Elevated levels of sugars have also been shown to induce the expression of several genes involved in plant defence. This includes the petunia chalcone synthase *ChsA* gene, which is involved in anthocyanin synthesis in response to UV or elicitors and whose expression is strongly induced by sucrose (Tsukaya *et al.*, 1991), and the wound-inducible potato proteinase inhibitor II gene (Johnson & Ryan, 1990). Other systems in which sucrose is capable of mediating an increase in gene expression include nitrate assimilation. Nitrate must be reduced to ammonia in order to be incorporated into amino acids. This reaction is catalysed by nitrate reductase (NR) which requires reducing power from NADH to reduce nitrate. NADH is itself derived from carbohydrate catabolism. Both light and sucrose induce accumulations of *Nr1*



mRNA in dark-adapted *A. thaliana* plants (Cheng *et al.*, 1992). This may reflect coordinate regulation of NR activity with photosynthesis, whereby nitrate reduction will only take place when sufficient carbohydrate is available to do so without overtaxing supplies.

Patatin, a major potato tuber protein, is encoded by a multigene family. The expression of a chimaeric gene consisting of the promoter of a class I patatin gene fused to the *gus* gene, is specifically induced by sucrose (Wenzler *et al.*, 1989). The class I patatin promoter can also direct expression of a chimaeric *gus* gene in leaves, where patatin is not normally synthesised, when plantlets are grown in high levels of sucrose (Rocha-Sosa *et al.*, 1989). The expression of genes encoding two tuberous root storage proteins in sweet potato (*Ipomoea batatas*), sporamin and  $\beta$ -amylase, can be induced in leaves and petioles, where they are not normally expressed, by high levels of sucrose, glucose and fructose (Hattori *et al.*, 1991; Nakamura *et al.*, 1991). The tissue specificity and sugar responsiveness of class I patatin gene expression are conferred by defined elements of the promoter (Grierson *et al.*, 1994). Within a segment of promoter called the proximal A+B repeat, a sequence of 17 bp is responsible for tissue specificity but can also confer a degree of sugar responsiveness in stem tissue. More proximal to the transcription start site, two sucrose response elements (SURE) of 9 bp each specifically confer a sucrose induction response. These sequences show close homology to the SP8 motif of the sweet potato sporamin gene, implicated in sucrose responsiveness (Ishiguro & Nakamura, 1992). A tuber protein binds to the SURE elements with similar binding specificity to a sweet potato factor, SP8F, which binds to the SP8 motif (Grierson *et al.*, 1994).

In the case described above (Grierson *et al.*, 1994), the genetic element conferring a developmental response (tissue specificity) and an element specifically involved in a metabolic response (sugar inducibility) can be distinguished. Additionally, in several of the cases described in this section (for example, Rocha-Sosa *et al.*, 1989 and Hattori *et al.*, 1991) an increase in levels of sucrose has been shown to overcome the signal which normally restricts expression of the genes in question to specific tissues.



### 1.5.2.2 Repression of gene expression

Sugars repress the expression of many genes involved in generating or maintaining levels of sucrose in source tissues. In higher plants, this type of regulation was first shown in a maize protoplast transient expression system, where it was found that the transcription of chimaeric genes consisting of the *cat* coding sequence under the control of the promoters of seven maize photosynthetic genes was repressed by sucrose, glucose (both photosynthetic end-products) and acetate (an exogenous carbon source) (Sheen, 1990). Repression by acetate in this system was greater than by sucrose or glucose, indicating a different mode of repression. In dark-adapted seedlings of *A. thaliana*, expression of the gene encoding the RuBisCO small subunit, *RbcS*, is induced by light, but sucrose represses this response (Cheng *et al.*, 1992). Addition of glucose to *Chenopodium rubrum* cell cultures leads to a rapid and reversible decrease of *RbcS* transcript levels (Krapp *et al.*, 1993). In rice, expression of the  $\alpha$ -amylase gene is very low in the presence of sucrose and is derepressed by sucrose starvation conditions (Yu *et al.*, 1991). An increase of  $\alpha$ -amylase synthesis under starvation conditions may be a means of accelerating hydrolysis of cellular starch as an energy source in response to the depletion of an exogenous carbon source.

Genes involved in the glyoxylate cycle and gluconeogenesis are subject to carbohydrate repression. This was first noted in cotyledons isolated from germinating castor bean (Lado *et al.*, 1967) where the normal increase in ICL activity at germination was found to be strongly reduced by the presence of 0.1 M glucose, although inhibition was not total. In anise suspension cultures (Kudielka & Theimer, 1983a,b), no MS or ICL activity was found when the growth medium contained sucrose. Removal of sucrose derepressed the activity of these enzymes. Addition of acetate further derepressed, or induced, activities. Addition of sucrose to a culture medium already containing acetate restored repression of MS and ICL, showing that the repression mediated by sucrose is a dominant factor in this system.



Similar effects have been observed in maize root tips under starvation conditions brought about by excision from the rest of the plant and incubation in a medium lacking sugars (Dieuaide *et al.*, 1992). This is effectively the same as treatments which have been carried out with the intention of simulating natural senescence and have been referred to as such by some workers (Gut & Matile, 1988), or have been used as an experimental approach to investigate expression of glyoxylate cycle genes (for instance, Graham *et al.*, 1992). Whole root tips deprived of sugars develop higher  $\beta$ -oxidation and MS (though not ICL) activities than in a medium providing glucose (Dieuaide *et al.*, 1992). As discussed in section 1.4.5, the expression of *Ms* and *Icl* is induced by treatments leading to starvation conditions in a variety of tissues, including detached and dark-incubated cotyledons, leaves and roots (Graham *et al.*, 1992; McLaughlin and Smith, 1994), whole mature plants incubated in darkness (Graham *et al.*, 1992), seedlings incubated in darkness (Sarah *et al.*, 1996; Reynolds & Smith, 1995a,b), protoplasts (McLaughlin and Smith, 1994) and cell cultures (Graham *et al.*, 1994a).

The induction of gene expression by starvation in these examples has in several instances been shown to be reversible on addition of sucrose. Expression of *Ms* and *Icl* in detached and dark-incubated cucumber cotyledons can be repressed by the addition of sucrose (McLaughlin and Smith, 1994), and accumulations of immunologically detectable MS and ICL (McLaughlin and Smith, 1994), *Icl* mRNA and GUS activity arising from *Icl* promoter-*gus* fusions (Reynolds & Smith, 1995b) in starved cucumber protoplasts are all repressible by sucrose. In whole transgenic *N. plumbaginifolia* seedlings containing *Icl* (Reynolds & Smith, 1995b) or *Ms* (Sarah *et al.*, 1996) promoter-*gus* fusions, the induction of GUS activity resulting from dark-incubation can also be repressed by the presence of sucrose. In cucumber callus cultures, starvation imposed by replacement of sucrose with mannitol in the culture medium led to a progressive accumulation of *Ms* and *Icl* transcripts, the rate and final extent of which was dependent on the concentration of sucrose in the medium prior to starvation (Graham *et al.*, 1994a). Expression of these genes in cultures pre-treated with mannitol was repressed when cells were transferred to media containing either sucrose, glucose, fructose or raffinose. Relative levels of expression of *Ms* and *Icl*



negatively correlated with intracellular sucrose levels and appeared to indicate a threshold level above which *Ms* and *Icl* expression was fully repressed. Repression did not occur with media containing succinate or malate. Intracellular levels of sugars and respiration rate in cultures maintained in media containing malate or succinate dropped as with mannitol, showing that intracellular sugar concentrations in these cultures could not be maintained by gluconeogenesis even though *Ms* and *Icl* were being expressed. This apparent dissociation between the glyoxylate cycle and gluconeogenesis under these conditions may suggest an anapleurotic role for the glyoxylate cycle in starvation, by providing succinate to replenish the carbon skeletons of the tricarboxylic acid cycle (Graham *et al.*, 1994a; McLaughlin & Smith, 1994; Kim & Smith, 1994b).

#### **1.5.2.3** *The responses of Ms and Icl gene expression to starvation and during post-germinative growth are separable*

The results of starvation experiments suggest the possibility that the observed synthesis of enzymes involved in the glyoxylate cycle concomitant with the onset of senescence may be a response to the metabolic status of the tissue in question rather than a programmed developmental event, as is the case for the process of leaf senescence itself. The genes encoding gMDH (*Mdh*) and PEPCK (*Pck*) are coordinately expressed with *Ms* and *Icl* during post-germinative growth in cucumber cotyledons (Kim & Smith, 1994a,b). However, in contrast to the inducibility of *Ms* and *Icl* expression by starvation and the repression of this response by sucrose, neither *Mdh* nor *Pck* are subject to such control. This suggests that expression of *Ms* and *Icl* during post-germinative growth is not mediated by sugars as is the case in starvation conditions (Kim & Smith, 1994a,b) and lends further support to the hypothesis that the glyoxylate cycle fulfills an anapleurotic role rather than gluconeogenic role during starvation (see section 1.3.1.4; Graham *et al.*, 1994a).

Further evidence supporting separate control of the post-germinative growth response and the direct metabolic response has been provided by detailed analyses of



the cucumber *Icl* (Reynolds & Smith, 1995b) and *Ms* (Sarah *et al.*, 1996) promoters directing *gus* expression in transgenic *N. plumbaginifolia*. Deletion of specific segments of the *Icl* promoter (Reynolds & Smith, 1995b) showed that a 572 bp segment of promoter proximal to the start of translation contained sufficient information to direct the correct spatial and temporal pattern of *gus* expression during post-germinative growth of transgenic seedlings, albeit at a lower level than constructs containing 2900 bp of promoter. However, the segment of promoter between 1663 and 1142 bp upstream of the start of translation was necessary for the sucrose response in transient expression assays in protoplasts. Though this does not exclude the possibility that more proximal sequences are also necessary for the carbohydrate response, it clearly indicates a physical separation of the elements responsible for mediating expression during post-germinative growth and in response to sucrose.

Similar results have been obtained for the *Ms* promoter (Sarah *et al.*, 1996). Deletion of 17 bp from a segment of the *Ms* promoter containing 199 bp of sequence upstream from the transcription start site results in almost complete abolition of the post-germinative growth response of *Ms* promoter-*gus* constructs in transgenic seedlings. Further deletion of 40 bp from the resulting 182 bp segment results in complete abolition. In contrast, constructs including more than 233 bp of promoter respond to starvation treatment and repression by sucrose, whilst constructs containing 216 bp or less are not starvation inducible. Thus, constructs containing 216 bp and 199 bp are both induced during post-germinative growth but are not responsive to starvation. The genetic elements mediating the two responses are therefore separate.

Sequence comparisons of the cucumber *Ms* and *Icl* promoters have revealed segments of homology (Sarah *et al.*, 1996). One such IMH (*Icl* - *Ms* homology) sequence within the segment of each gene implicated in carbohydrate control, IMH2, shows similarity with Myb binding sequences in other plant genes (Grotewold *et al.*, 1994). Another sequence, IMH5, within the 17 bp segment of *Ms* implicated in the post-germinative growth response and present in the opposite orientation in the *Icl*



promoter, shows homology with *amd19*-like sequences from *A. nidulans* and *Neurospora crassa* which are thought to be binding sites for the *facB* gene product (see section 1.5.1; Sandeman *et al.*, 1991). This may suggest the involvement of acetate or acetyl CoA from lipid breakdown in the post-germinative regulation of *Ms* and *Icl* (Graham *et al.*, 1992). Another sequence, IMH1, appears to specifically bind a factor(s) from cucumber callus extract (Graham *et al.*, 1994b).

#### 1.5.2.4 Sugar sensing and signal transduction

Although the detailed mechanism of sugar sensing in higher plants remains to be elucidated, evidence suggests the involvement of hexokinase in the initial sensing step leading to the repression of glyoxylate cycle genes. In cucumber cell cultures, whereas the presence of glucose, fructose or sucrose results in repression of *Ms* and *Icl* expression (see previous section), repression did not occur with 3-*O*-methylglucose, a non-metabolisable analogue of glucose (Graham *et al.*, 1994a). This compound is taken up by cells, but is not phosphorylated by hexokinase. Similar results have been obtained in protoplast transient assays (Graham *et al.*, 1994b). Mannose and 2-deoxyglucose, which can be phosphorylated by hexokinase but are not further metabolised, cause repression even though intracellular concentrations of sucrose, glucose and fructose fall below the threshold level at which derepression occurs under normal circumstances. These results implicate hexokinase in the initial sugar sensing step and suggest that further metabolism of sugar phosphates is not necessary for this response.

In *Chenopodium rubrum* cell cultures (Krapp *et al.*, 1993), glucose analogues 3-*O*-methylglucose and 6-deoxyglucose (which, like 3-*O*-methylglucose, is transported into cells but is not phosphorylated or further metabolised) fail to have an effect on *RbcS* transcript levels, whilst glucose causes severe repression when supplied at the same concentration. This suggests that the metabolism of glucose is necessary for it to cause repression. Transient expression studies using the *cat* reporter gene under the control of the promoters of three photosynthetic genes in



maize protoplasts (Jang & Sheen, 1994) have shown various hexoses and 2-deoxyglucose to cause repression whilst 3-*O*-methylglucose does not. Furthermore, a competitive inhibitor of hexokinase, mannoheptulose, relieves the repression caused by glucose and 2-deoxyglucose. Taken together, these results strongly support the role of hexokinase in mediating the described repression of gene expression caused by sugars, as appears to be the case in *S. cerevisiae* (refer to section 1.5.1).

The signal transduction pathway leading to regulation of gene expression by sugars has yet to be elucidated. Higher plant homologues of the *S. cerevisiae* *SNF1* gene have been cloned (Halford *et al.*, 1992; Le Guen *et al.*, 1992) and one of these is able to complement the *S. cerevisiae* *snf1* mutant (Alderson *et al.*, 1991).

## **1.6 Antisense inhibition of gene expression**

The research project described in this thesis made use of antisense gene techniques in an attempt to inhibit the expression of the *Ms* and *Icl* genes in transgenic plants. Antisense technology has been widely used in research but the mechanism of inhibition is not fully understood. This section will briefly discuss some important features of antisense technology.

### **1.6.1 Applications of antisense technology**

Antisense RNA was initially recognised as a naturally occurring mechanism for regulating plasmid replication and gene expression in bacteria (Green *et al.*, 1986). Although there is some evidence supporting the existence of naturally occurring antisense RNA in plants (Rogers, 1988), the majority of experimental evidence concerning the mechanism of antisense genes has been accumulated from artificial systems in which transgenes have been employed to repress the expression of endogenous genes. The use of antisense genes to inhibit gene expression in plants was first demonstrated with the (bacterial) *cat* gene in carrot protoplasts (Ecker &



Davis, 1986). Antisense inhibition of endogenous plant genes was subsequently demonstrated in two important cases. The first of these was antisense inhibition of chalcone synthase (CHS) synthesis in petunia (van der Krol *et al.*, 1988). CHS is involved in flower pigmentation. A *ChsA* cDNA was fused in the antisense orientation with respect to the cauliflower mosaic virus (CaMV) 35S promoter and the resulting plasmid introduced into petunia. In a significant proportion of transformants regenerated, the levels of endogenous *Chs* mRNA and protein were reduced, in some cases severely, and flower pigmentation in many transformants was effectively inhibited. The degree of inhibition appeared to correlate with levels of antisense RNA. The second of these early endogenous gene inhibition experiments was the inhibition of polygalacturonase (PG, involved in fruit softening) gene expression in transgenic tomatoes (Smith *et al.*, 1988). Applications of antisense technology have since been wide-ranging and have included analysis of metabolic pathways, identifying gene functions and crop improvement (for review, see Bourque, 1995).

### **1.6.2 Components of antisense genes**

#### **1.6.2.1 Coding sequences**

Chimaeric antisense genes consist of essentially two components, a DNA segment derived from an endogenous, heterologous or foreign gene, and a promoter to which the DNA segment is fused in the antisense orientation with the purpose of directing transcription of the DNA segment. Antisense genes have been constructed with a variety of DNA sequences. Although endogenous genomic sequences have been used (for example, Kuipers *et al.*, 1995), chimaeric antisense genes have most commonly been constructed with cDNA sequences derived from endogenous genes. This includes complete cDNA sequences (for example, van der Krol *et al.*, 1988, 1990a; Sandler *et al.*, 1988; Kuipers *et al.*, 1995) and partial cDNA sequences. The partial segment of the coding region used has varied widely and there does not appear to be any single part of a cDNA which is consistently more effective than any other in causing inhibition. Segments derived from 5' regions of cDNAs (for example, Smith *et al.*, 1988; Flachmann & Kühlbrandt, 1995; Kuipers *et al.*, 1995), central regions



(for example, Sandler *et al.*, 1988; van der Krol *et al.*, 1990a) and 3' regions (for example, van der Krol *et al.*, 1990a) have all been successful in causing antisense inhibition. In addition, antisense inhibition of endogenous genes with homologues isolated from other species has been demonstrated (for example, Oliver *et al.*, 1993) and of transgenes in plants (for example, Guerineau *et al.*, 1994; Bourque & Folk, 1992) has been achieved. These aspects of chimaeric antisense gene construction will be more extensively discussed in section 5.1.1.

#### **1.6.2.2 Promoters**

Transcription of chimaeric antisense genes in transgenic plants has most often been directed from the nominally constitutive CaMV 35S promoter (Bourque, 1995). Exceptions include use of the *Chs* promoter to direct transcription of an antisense petunia *Chs* cDNA fragment (van der Krol *et al.*, 1990a) and the granule-bound starch synthase (GBSS) promoter to direct expression of an antisense *Gbss* cDNA fragment (Kuipers *et al.*, 1995). In both these cases, promoter and cDNA were derived from the same species as the transgenic host. In other cases, promoters have been selected with the intention of restricting expression to, or enhancing expression within, specific tissues. Examples of this include the use of the potato *St-ls1* promoter (active in photosynthetic tissues only) to direct transcription of a potato antisense *Agpase* cDNA in that species (Leidreiter *et al.*, 1995), use of the light-regulated *Cab* promoter from *A. thaliana* to direct transcription of an antisense *gus* gene to inhibit the expression of a sense *gus* transgene in transgenic *N. plumbaginifolia* (Cannon *et al.*, 1990) and modification of the CaMV 35S promoter by addition of multiple copies of the petunia anther-box motif to extend the activity of this promoter to anther tissues, where it otherwise has a low level of activity, in order to direct transcription of a petunia *Chs* antisense cDNA in that species (van der Meer *et al.*, 1992).

#### **1.6.3 Mechanism of antisense inhibition**

The mechanism by which chimaeric antisense genes inhibit expression of their endogenous counterparts is not fully understood. From studies of naturally occurring



antisense regulation in bacteria (Green *et al.*, 1986), it is thought that synthesis of antisense RNA is necessary for inhibition and that the antisense RNA probably interferes with translation by annealing to its sense counterpart and blocking ribosome binding and translational initiation. The rate of transcription of the endogenous gene does not appear to be affected in plants whose steady-state endogenous mRNA level has been reduced by the synthesis of antisense RNA (Sheehy *et al.*, 1988). With the use of chimaeric antisense genes in plants, it is generally accepted that sequence complementarity between the antisense RNA and the target gene is a critical element (Bourque, 1995). If the degree of homology between the antisense and target genes is compromised, this appears to lead to less effective inhibition (Oliver *et al.*, 1993; Visser *et al.*, 1991). Introduction of a chimaeric antisense gene into plants generally results in transformants exhibiting a range of degrees of inhibition, attributed to effects related to the site of insertion of the transgene in each transformant, which tend to be stably inherited (Bourque, 1995).

Studies in which the steady-state levels of antisense RNA have been measured, reduction of endogenous mRNA is associated with the presence of antisense RNA (van der Krol *et al.*, 1988; Smith *et al.*, 1988; Kuipers *et al.*, 1995; Robert *et al.*, 1989). In some cases (Robert *et al.*, 1989; Delauney *et al.*, 1988) the levels of sense and antisense RNA appear to be inversely correlated, suggesting a mechanism whereby sense and antisense transcripts anneal to one another and either the translation of the mRNA is prevented or the hybrid molecule processed. If such a mechanism of pairing were operating, a stoichiometric excess of antisense RNA would clearly be expected to be more effective at causing inhibition. However, an inverse correlation is not always observed and an excess of antisense RNA is not a prerequisite for inhibition (Stockhaus *et al.*, 1990; van der Krol *et al.*, 1990a; Cannon *et al.*, 1990). This suggests that although the mechanism described may operate in some cases, alternative mechanisms must also exist. A comparison of the steady-state levels of endogenous *Chs* mRNA and a distinguishable (also endogenous) *Chs* sense mRNA species transcribed from the CaMV 35S promoter in a range of transgenic plants (van der Krol *et al.*, 1990a) showed that even in the transformant with the



highest steady-state level of the transgenic transcript, this level did not exceed that of the endogenous *Chs* genes. This suggests that the inhibition of *Chs* gene expression observed in plants containing antisense *Chs* genes, under the control of the CaMV 35S promoter, is not attributable to the greater strength of the CaMV 35S promoter and therefore a stoichiometric excess of antisense over sense transcripts. Synthesis of the 10 kDa photosystem II polypeptide in transgenic potato (Stockhaus *et al.*, 1990) was efficiently inhibited by a chimaeric antisense gene in some transformants, but no consistent correlation between antisense RNA levels and inhibition of endogenous mRNA levels was found. In another study (Flachmann & Kühlbrandt, 1995), despite the effective reduction of *Cab* mRNA caused by the introduction of an chimaeric antisense counterpart, the degree of reduction of mRNA in transformants appeared to be inversely correlated to steady-state levels of antisense RNA (this would, however, be consistent with simultaneous processing of sense and antisense transcripts in a double-stranded RNA hybrid). The stoichiometric antisense RNA - sense RNA interaction model is also put into question by the failure to detect the putative RNA duplex (Mol *et al.*, 1989; van der Krol *et al.*, 1990b). In view of these data, such a model alone seems unable to explain antisense inhibition, but could still apply if interactions between sense and antisense transcripts were transitory and a single antisense RNA molecule were capable of preventing the normal processing of several sense transcripts (Nellen & Lichtenstein, 1993).

As with steady-state transcript levels, there appears to be no consistent correlation between gene dosage and antisense inhibition. Homozygous progeny of PG antisense transformants showed a higher degree of inhibition than their heterozygous parents (Smith *et al.*, 1990). Another study showed that the degree of antisense inhibition appeared to depend on the number of copies of the antisense gene (Hamilton *et al.*, 1990). In contrast, pectin esterase activity in transgenic tomato plants was no more inhibited in plants homozygous with respect to the antisense gene than in heterozygotes (Hall *et al.*, 1993).

Effects on protein levels expressed from inhibited target genes or corresponding enzyme activities vary. Inhibition of the *Cab* gene in transgenic



*Nicotiana tabacum* resulted in effective reduction of mRNA but polypeptide levels appeared unchanged (Flachmann & Kühlbrandt, 1995), suggesting a regulatory mechanism compensating for the reduction of mRNA. Introduction of a chimaeric antisense cucumber *Hpr* gene into transgenic *N. tabacum* resulted in an effective reduction of HPR enzyme activity and protein levels but mRNA steady-state levels were unaffected (Oliver *et al.*, 1993). This suggests a mechanism which interferes with translation but does not involve degradation of mRNA. This could operate through the formation of a stable sense-antisense duplex which would either prevent export from the nucleus or directly prevent translation whilst not reducing detectable levels of mRNA (Oliver *et al.*, 1993; Bourque, 1995).

The elucidation of antisense inhibition is further confused by the phenomenon of co-suppression, whereby chimaeric sense constructs have been found to cause inhibition effects similar to antisense constructs (Napoli *et al.*, 1990; van der Krol *et al.*, 1990c). Although co-suppression effects show associated reductions of both endogenous and transgene steady-state RNA levels (Napoli *et al.*, 1990; Van Blokland *et al.*, 1994) this reduction has in one case (Van Blokland *et al.*, 1994) been shown not to be due to a reduction of transcriptional activity of the endogenous gene. Furthermore, in this study a promoterless construct was able to cause efficient inhibition, putting into question the necessity for transgenes to be transcribed at all in order to cause co-suppression. Antisense transcripts (in sub-stoichiometric amounts) were detected in transformants, suggesting the possibility that co-suppression could be mediated by antisense RNA resulting from aberrant transcription from flanking DNA (Grierson *et al.*, 1991; Van Blokland *et al.*, 1994). However, co-suppression effects have been found to be reversible, suggesting the involvement of DNA methylation (Napoli *et al.*, 1990; Neuhuber *et al.*, 1994). Suppression has been shown to be associated with methylation of both the endogenous gene and the transgene in some systems (Neuhuber *et al.*, 1994; Matzke *et al.*, 1994; Ingelbrecht *et al.*, 1994). However, in one of these studies (Ingelbrecht *et al.*, 1994), although co-suppression was associated with DNA methylation, this did not appear to affect transcriptional activity of the target gene. These workers concluded that methylation was therefore mediating suppression at the post-transcriptional level.



As is the case for antisense inhibition, it would appear that there is more than one mechanism by which co-suppression operates. Although the phenomena of antisense inhibition and co-suppression have been given distinct terms, the mechanisms by which they operate have not been sufficiently characterised to be able to clearly distinguish between the two. They may therefore share mechanisms of inhibition, or elements of these.

#### **1.6.4 *Inhibition of genes involved in metabolic pathways***

The project described in this thesis was an attempt to inhibit the synthesis of MS and ICL by the use of antisense techniques, in order to prevent the functioning of the glyoxylate cycle. It was anticipated that this might affect germination, post-germinative growth, pollen development, embryogenesis, senescence or the response to starvation. This approach makes the assumption that the inhibition of the individual steps catalysed by MS and ICL will influence the overall metabolic flux of the pathway. The change of flux of a metabolic pathway in response to a change in the activity of one component enzyme is a product of the interaction of all the steps in the pathway. It is therefore not sufficient to know the extent of a local (single enzyme) change in order to predict the effect on the overall flux. The overall response of a pathway to a change in the activity of a single specified enzyme is given by the flux control coefficient (Kacser & Porteous, 1987). Although the flux control coefficient refers to a single step, it is dependent on all the changes in metabolite concentrations in the pathway. It is therefore a systemic property and not one unique to any given enzyme. Thus, the assumption that the flux of a pathway will react in a linear way to changes in enzyme activity cannot be made (Kacser & Porteous, 1987).

Despite this limitation, inhibition of specific enzymes in metabolic pathways with antisense genes has been extensive. Notable examples include alteration of flavonoid biosynthesis with antisense *Chs* genes (van der Krol *et al.*, 1988) and dissection of the process of tomato fruit ripening (Smith *et al.*, 1988; Gray *et al.*,



1992. Refer to section 1.6.1 and examples within section 1.6.2). Antisense techniques have been successfully used to alter metabolic partitioning in a wide variety of cases (Herbers & Sonnewald, 1996). Incomplete inhibition with antisense genes can be a potential advantage in such studies, particularly if complete inhibition of a given gene is lethal. The range of degrees of inhibition often exhibited in sets of plants transformed with antisense genes is also a powerful tool for observing metabolic fluxes in response to partial reductions in the activities of enzymes involved in metabolic pathways. This is exemplified by the inhibition of RuBisCO activity with chimaeric antisense *RbcS* genes. A set of plants transformed with an antisense *RbcS* gene (Rodermeil *et al.*, 1988), exhibiting a progressive decrease in the amount of RuBisCO, has allowed detailed studies of the impact of a reduction in this enzyme on growth and metabolism (Rodermeil *et al.*, 1988; Quick *et al.*, 1991a), the control RuBisCO exerts on photosynthesis as a whole (Quick *et al.*, 1991b) and the regulation of RuBisCO activity in response to external conditions and metabolism (Stitt *et al.*, 1991). Antisense technology is therefore currently the most appropriate tool for examining the role and regulatory aspects of specific enzymes in plants.



## **2. MATERIALS AND METHODS**



**NOTE:** Unless stated otherwise, all chemical reagents were purchased from Sigma Chemical Co. Ltd. or BDH Chemicals Ltd. and solutions of the appropriate concentration were made by dissolving reagents in sterile double-distilled water (sddH<sub>2</sub>O). Sterilisation of media, chemicals and equipment was performed by either autoclaving (120 °C, 20 min, 15 lb in<sup>-2</sup>), baking (180 °C for a minimum of 6 h) or using disposable filters (0.2 µm pore size, Acrodisc PF, Gelman Sciences, Michigan).

## **2.1 Biological material**

### **2.1.1 *Plant material***

Potato tubers (*Solanum tuberosum* L. cv 'Desiree') were obtained commercially and allowed to sprout in Levington 3M potting compost in greenhouse conditions. Plants were grown to maturity, fruits collected and seed harvested from these. Sterile nodal cultures for transformation were obtained from A. Taylor, University of Aberdeen. Dihaploid *Nicotiana plumbaginifolia* seed was obtained from J. R. Ellis, Department of Botany, University of Durham. For seed germination, potato and *N. plumbaginifolia* seeds were surface-sterilised in 10 % (v/v) sodium hypochlorite for 15 min, washed 5 times in sddH<sub>2</sub>O and imbibed in 1 mM gibberellic acid for 48 h at 4 °C. Seeds were then washed again and sown on three different surfaces depending on subsequent use:

i) For growth of seedlings for harvesting at specific stages of post-germinative development, seeds were sown on 3 layers of moist 7 cm diameter Whatman no.1 filter paper circles in petri dishes sealed with Parafilm (American Can Co.) and germinated in constant irradiance of 120 µmol m<sup>-2</sup> s<sup>-1</sup> at 25 °C.

ii) For selection of kanamycin-resistant transgenic seedlings, seeds were sown on petri dishes containing 0.5 x M & S medium (2.35 g l<sup>-1</sup> Murashige & Skoog medium [Flow Laboratories, Irvine.], 0.8 % [w/v] bacto-agar [Difco laboratories]) and 200 µg ml<sup>-1</sup> kanamycin and grown in the same conditions as above.



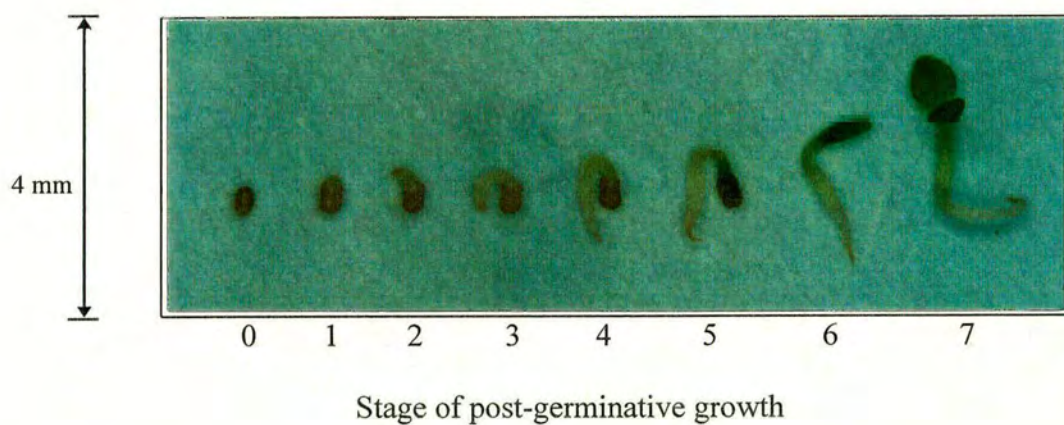
iii) For growth of seedlings to maturity, seeds were sown in Levington 3M compost and incubated in a regime of 14 h light at 25 °C with an irradiance of 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (metal halide lamps) and 10 h dark at 20 °C.

Stages of post-germinative growth in potato and *N. plumbaginifolia* were specified according to the length of radicle compared to the seed coat and the apparent maturity of the cotyledons. These stages in *N. plumbaginifolia* are illustrated in figure 2.1 and are defined as follows:

- Stage 0: Seed immediately following imbibition.
- Stage 1: Seed coat split open, radicle clearly emerging.
- Stage 2: Radicle curling back towards seed, tip at same level as split in seed-coat.
- Stage 3: Radicle approximately same length as seed coat.
- Stage 4: Radicle approximately twice the length of the seed coat.
- Stage 5: Radicle length at least three times length of seed coat, cotyledons green but still within seed coat.
- Stage 6: Cotyledons fully emerged from seed coat but still separating and expanding.
- Stage 7: Cotyledons completely separated and fully expanded.

Seedlings were selected with the aid of a binocular microscope at 10 x magnification, using a pair of fine forceps and immediately frozen in liquid nitrogen. *N. plumbaginifolia* anthers collected for total RNA extraction were harvested from flowers where the length of the carpels was equal to the length of the petals, which were unopened at this stage. The petals were slit open with a scalpel, the anthers removed with a pair of fine forceps and immediately frozen in liquid nitrogen. For cross-pollination experiments, flowers at this stage were emasculated by removal of anthers with a pair of fine forceps and the stigma brushed with dehiscent anthers removed from more mature flowers with opened petals.





**Figure 2.1:**

**Stages of post-germinative growth in *N. plumbaginifolia***

Numbers correspond to stages defined in text and do not necessarily correspond to days after imbibition, except for stage 0 which is defined as seed immediately following imbibition.



2.1.2 Bacterial strains and genotypes

*Escherichia coli*:

XL1-Blue                    *supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac<sup>-</sup> F'*  
                                  [*proAB<sup>+</sup> lacIQ lacZ ΔM15 Tn10 (tet<sup>r</sup>)*]  
Used as a host for recombinant manipulation.

DH5α                        *supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1*  
                                  *gyrA96 thi-1 relA1*  
Used as a host for recombinant manipulation.

*Agrobacterium tumefaciens*:

LBA4404                    Genotype not available. Carries both a cryptic and a disarmed  
Ti plasmid, the latter lacking the entire T-DNA segment, but  
with an intact *vir* region. The bacterial chromosome carries  
streptomycin resistance and the disarmed Ti plasmid carries  
rifampicin resistance (Hoekema *et al.*, 1983)

2.1.3 Bacterial plasmids

Vector	Source	Use
pBluescript II SK+	Stratagene	Cloning of PCR products, subcloning, nucleotide sequence determination.
pUC19	Yanisch-Perron <i>et al.</i> (1985)	subcloning.
pBI121	Jefferson <i>et al.</i> (1987)	Plant transformation.



#### 2.1.4 *Cucumber (Cucumis sativus L) isocitrate lyase partial cDNA clone*

The pBS+ (Stratagene) plasmid containing a 1.4 kbp cDNA fragment partially encoding cucumber *Icl*. Designated pBSICL (Reynolds & Smith, 1995a).

#### 2.1.5 *Cucumber malate synthase cDNA clone*

The pBS+ plasmid containing a 1.9 kbp cDNA fragment encoding cucumber *Ms*. Designated pBSMS1.9 (Graham *et al.*, 1989).

#### 2.1.6 *Restriction endonucleases and DNA modification enzymes*

All restriction endonucleases and DNA modification enzymes were purchased from Northumbria Biologicals Ltd. (NBL), Boehringer Mannheim Biochemica, Pharmacia LKB or Gibco-BRL.

#### 2.1.7 *Culture of bacterial strains*

Using standard sterile techniques, single bacterial colonies were picked using a wire loop and used to inoculate appropriate volumes of Luria-Bertani medium (LB, pH 7, 10 g l<sup>-1</sup> bacto-tryptone [Difco], 5 g l<sup>-1</sup> bacto-yeast extract [Difco], 10 g l<sup>-1</sup> NaCl) containing the appropriate antibiotics. Cultures were grown by shaking at 37 °C (*E. coli*) or 30 °C (*A. tumefaciens*). For isolation of single bacterial colonies by streaking-out or plating of bacterial transformation mixes, LB agar was used (as LB medium but with the addition of 15 g l<sup>-1</sup> bacto-agar) containing the appropriate antibiotic. Antibiotics for bacterial culture were used at the following concentrations (DMSO: dimethylsulfoxide):

Antibiotic	Stock solution	Working concentration
Ampicillin	100 mg ml <sup>-1</sup> in sddH <sub>2</sub> O	100 µg ml <sup>-1</sup>
Kanamycin	100 mg ml <sup>-1</sup> in sddH <sub>2</sub> O	200 µg ml <sup>-1</sup>
Rifampicin	100 mg ml <sup>-1</sup> in DMSO	100 µg ml <sup>-1</sup>
Streptomycin	300 mg ml <sup>-1</sup> in sddH <sub>2</sub> O	300 µg ml <sup>-1</sup>
Tetracycline	5 mg ml <sup>-1</sup> in ethanol	10 µg ml <sup>-1</sup>



## 2.2 Nucleic acid isolation, manipulation and analysis

### 2.2.1 Extraction of total RNA from potato and *N. plumbaginifolia* seedlings

This procedure was carried out at 4 °C. For extraction of total RNA for subsequent reverse-transcription and polymerase chain reaction (PCR) amplification, potato and *N. plumbaginifolia* seedlings were harvested at five days post-imbibition and homogenised with a sterile mortar and pestle. For extraction of total RNA for northern analysis, seedlings at specific developmental stages were harvested as described in section 2.1.1 and homogenised in a sterile microfuge tube with a micro-pestle (Treff Lab) in extraction buffer (100 mM Tris-HCl pH 8.5, 1 % [w/v] triisopropylmethylphthalene sulphonate [TMS], 6 % [w/v] 4-aminosalicylic acid [PAS]), where 1 ml of extraction buffer was added per g of seedling material and a minimum of 0.5 ml for small quantities of material. An equal volume of phenol (buffered to pH 7.4 with 1 M Tris- HCl) was immediately added and the sample thoroughly mixed. The organic and aqueous phases were separated by centrifugation at 12000 g for 5 min and the supernatant removed to a fresh tube. Nucleic acids remaining in the phenol or interface were further extracted by addition of an equal volume of extraction buffer to the phenol, followed by mixing and centrifugation again. The resulting aqueous phase was removed and added to that from the first extraction. An equal volume of phenol was added to the pooled supernatants, mixed and centrifuged, the new aqueous phase removed and the process repeated with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) to remove traces of phenol. Total nucleic acid in the resulting supernatant was precipitated by addition of 0.1 volumes of 3 M sodium acetate (pH 5.5) and 2 volumes of ethanol followed after 1 h at -20 °C by centrifugation at 12000 g for 15 min. The resulting pellet was redissolved in  $\text{sddH}_2\text{O}$ , an equal volume of 5 M lithium chloride added to specifically precipitate total RNA, the mixture left at -20 °C overnight and RNA pelleted by centrifugation at 12000 g for 20 min at 4 °C. The pellet was redissolved in  $\text{sddH}_2\text{O}$  and precipitation with sodium acetate and ethanol, as described above, repeated. The final pellet was washed with 70 % (v/v) ethanol, dried under vacuum and redissolved in  $\text{sddH}_2\text{O}$  treated with diethylpyrocarbonate (DEPC, 0.05 % v/v, 65 °C for 12 h and





autoclaved for 30 min) to inhibit contaminating ribonucleases. The purity and yield of RNA was assessed by spectrophotometric scan between wavelengths of 220 nm and 320 nm using a Beckman DU-64 spectrophotometer. An  $A_{260}$  reading of 1 corresponds to approximately  $40 \mu\text{g ml}^{-1}$  of RNA and an estimate of sample purity is provided by the ratio of the absorbance value at 260 nm to that at 280 nm, pure RNA having a value of 2.0.

### **2.2.2 *Horizontal gel electrophoresis of RNA***

RNA samples were size-fractionated by electrophoresis through gels prepared from agarose (ultra-pure) at a concentration of 1.4 % (w/v) in 1 x MOPS buffer (20 mM 3-[N-morpholino]propanesulfonic acid [MOPS], 5 mM sodium acetate pH 7.0, 1 mM EDTA pH 8.0). The agarose was melted by boiling in 1 x MOPS buffer and the volume of the solution reduced to allow addition of formaldehyde to a final concentration of 6.6 % (w/v). This was added when the agarose had been allowed to cool at room temperature for 5 min.

Samples were prepared for electrophoresis by dessicating aliquots to complete dryness under vacuum and redissolving the RNA in 15  $\mu\text{l}$  of RNA gel loading buffer (1 x MOPS buffer, 50 % [v/v] formamide, 6.6 % [w/v] formaldehyde, 3 % [w/v] Ficoll 400, 0.02 % [w/v] bromophenol blue, 50  $\mu\text{g ml}^{-1}$  ethidium bromide). Samples were heated at 70 °C for 5 min immediately prior to loading. The RNA was visualised using a short wavelength trans-illuminator (Hybaid). RNA size markers were purchased from Gibco BRL and included 6 standard size markers from 0.24 to 9.5 kb.

### **2.2.3 *Northern (RNA) blotting***

After fractionation and visualisation of RNA, gels were photographed and the RNA transferred to Hybond N filters (Amersham International plc.) by capillary action according to the method of Southern (1975). Transfer was facilitated by imposing a salt gradient across the gel from a reservoir of 20 x SSPE (3.6 M NaCl, 0.2 M sodium dihydrogen orthophosphate pH 7.7, 20 mM EDTA pH 8.0) to the filter,



which had been soaked in 2 x SSPE. After transfer, the filters were rinsed in 2 x SSPE, baked at 60 °C for 15 min and the RNA crosslinked to the nylon membrane by ultra-violet (UV) irradiation at 0.4 J cm<sup>-2</sup> using a Hybaid crosslinker.

#### 2.2.4 Radiolabelling of ds DNA probes by random priming

Double-stranded DNA was labelled by the random priming method of Feinberg & Vogelstein (1983). The template for *Ms* probe synthesis was the fragment of pBlSc TMS6 (section 2.4.5) excised when digested with *Xba*I and *Xho*I and that for *Icl* probe synthesis was the fragment of pBlSc TICL10 (section 2.4.5) excised when digested with *Eco*RI and *Sal*I. 50 ng template DNA was denatured by heating at 100 °C for 3 min and then chilled on ice. An appropriate volume of ddH<sub>2</sub>O was then added to the denatured DNA to result in a final volume of 50 µl when the following were added:

5 x oligonucleotide labelling buffer (OLB)	10 µl
BSA (10 mg ml <sup>-1</sup> )	2 µl
[α- <sup>32</sup> P]dCTP	3 µl (≡ 30 µCi)
DNA polymerase I Klenow fragment	1 µl (≡ 1 U)

[α-<sup>32</sup>P]dCTP was purchased from Amersham International plc. 5 x OLB: 250 mM Tris-HCl pH 8.0, 25 mM MgCl<sub>2</sub>, 5 mM 2-mercaptoethanol, 2 mM with respect to each of dATP, dGTP and dTTP, 1 M HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), adjusted to pH 6.6 with 4 M sodium hydroxide, 1 mg ml<sup>-1</sup> random hexanucleotides. The labelling reaction was allowed to proceed at room temperature overnight.

The reaction was stopped by addition of 200 µl Stop buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA pH 8.0, 0.5 % [w/v] SDS) and unincorporated nucleotides removed by ion-exchange using an ELUTIP-d column (Anderman Co. Ltd.) according to the manufacturer's instructions. Immediately prior to use, the radiolabelled DNA was denatured by heating at 100 °C for 3 min.



### 2.2.5 Hybridisation of radiolabelled DNA probes to membrane-bound RNA

After UV crosslinking, membrane-bound RNA was pre-hybridised in 50 ml hybridisation buffer (4 x SSPE, 20 mM Tris-HCl pH 7.6, 1.0 % [w/v] SDS, 2 x Denhardts solution [1 % (w/v) bovine serum albumin fraction V, 1 % (w/v) Ficoll 400, 1 % (w/v) polyvinylpyrrolidone  $M_r$  40000], 10 % [w/v] dextran sulphate) for at least 2 h at 65 °C in glass tubes in a rotisserie oven (Hybaid). After pre-hybridisation, the solution was replaced with 10 ml of fresh hybridisation buffer to which the denatured radiolabelled probe had been added and the filters hybridised overnight at 65 °C. After hybridisation, the filters were passed through successively more stringent (lower salt concentration) washes as follow:

Wash	Solution	Conditions
Wash A	4 x SSPE, 1.0 % (w/v) SDS	65 °C, 45 min x 2 washes
Wash B	2 x SSPE, 0.5 % (w/v) SDS	25 °C, 30 min x 2 washes
Wash C	2 x SSPE	25 °C, 30 min x 2 washes

After washing, the filters were partially air dried and wrapped in Saran wrap.

### 2.2.6 Synthesis of digoxigenin (DIG) -labelled ss RNA probes

Single-stranded RNA probes (riboprobes) labelled with DIG were synthesised by *in vitro* transcription using linearised pBlScTMS6 and pBlScTICL10 (section 2.4.5) as templates. T7 and T3 RNA polymerases (Boehringer Mannheim) were used to transcribe from their respective promoters on the vectors in the presence of DIG-NTP labeling mix (Boehringer Mannheim) according to the manufacturer's instructions. UTP residues conjugated to DIG are incorporated into the newly-synthesised RNA strand by this process. The templates used for riboprobe synthesis were as follow:



Template vector	Restriction endonuclease used to linearise template	Promoter initiating transcription	Target strand of probe
pBlSc TMS6	<i>Pst</i> I	T3	sense
pBlSc TMS6	<i>Bam</i> HI	T7	antisense
pBlSc TICL10	<i>Sal</i> I	T3	sense
pBlSc TICL10	<i>Pst</i> I	T7	antisense

For each *in vitro* transcription reaction, approximately 1.2 µg of template DNA was used with 40 U of either T3 or T7 RNA polymerase. Purification of labelled RNA was carried out according to the manufacturer's recommendations and the yield of RNA from the reaction was estimated by agarose gel electrophoresis (section 2.2.2).

### 2.2.7 Hybridisation of DIG-labelled riboprobes to membrane-bound RNA

After RNA samples were fractionated by electrophoresis (section 2.2.2) and immobilised on nylon membrane (section 2.2.3), hybridisation with DIG-labelled riboprobes was carried out using 50 ng of riboprobe (denatured by heating at 100 °C for 5 min) in a total volume of 20 ml hybridisation buffer (5 x or 2 x SSPE, 0.1 % [w/v] N-laurylsarcosine, 0.02 % [w/v] SDS, 1 % [w/v] Boehringer Mannheim DIG Luminescent Detection Kit Blocking reagent). Hybridisation conditions were as follow, with a prehybridisation of at least 1 h in the same buffer as was used for the hybridisation, which was itself allowed to proceed for at least 12 h on a shaking platform in a Hybaid oven:

#### HYBRIDISATION:

	'Low stringency'	'High stringency'
Concentration of SSPE in hybridisation buffer	5 x	2 x
Temperature of hybridisation	70 °C	72 °C



## STRINGENCY

### WASHES:

	'Low stringency'	'High stringency'
<b>Wash A (2 x 30 min):</b>		
Concentration of SSPE	2 x	0.1 x
Temperature	70 °C	72 °C
<b>Wash B (2 x 30 min):</b>		
Concentration of SSPE	0.1 x	0.1 x
Temperature	room temperature	room temperature

Wash A also contained 0.1 % (w/v) SDS. Each filter underwent one 'low stringency' hybridisation and set of washes and at least one (up to 3) 'high stringency' hybridisation and set of washes. In between each set of hybridisation and washes, the filters were incubated at 75 °C in 0.2 % (w/v) SDS on a shaking platform for 2 h.

### 2.2.8 Luminescent detection of DIG-labelled riboprobes

Riboprobes hybridised to membrane-bound RNA were detected using the Boehringer Mannheim DIG Luminescent Detection Kit according to the manufacturer's instructions. This enables immunological detection of DIG with an antibody conjugated to alkaline phosphatase, which catalyses a chemiluminescent reaction to which X-ray film is sensitive.

### 2.2.9 Isolation of plasmid DNA from *E. coli* and *A. tumefaciens*

Small-scale (volumes of bacterial culture up to 10 ml) and large-scale (greater than 10 ml) extractions of plasmid DNA from *E. coli* and *A. tumefaciens* host strains were carried out according to the method of Sambrook *et al.*, 1989, but with the following modifications:



i) Instead of fixed volumes of Solution I (cell resuspension buffer) being used, the volume chosen was always 0.1 x that of the culture used and the volumes of reagents used subsequently were adjusted accordingly.

ii) Cellular debris and chromosomal DNA in the solution resulting from alkaline lysis were initially precipitated by addition of 0.5 volumes of 3 M sodium acetate, pH 5.0. Protein in the resulting supernatant was removed by two phenol extractions. Phenol traces in the supernatant resulting from the phenol extractions were removed by addition of an equal volume of chloroform:isoamyl alcohol.

iii) Plasmid DNA was precipitated by addition of 2 volumes of ethanol.

iv) For large scale preparations of plasmid DNA or preparations from which nucleotide sequences were to be determined, the following steps were added to the extraction protocol: after dissolving the final pellet of DNA and treatment with RNase, two further phenol extractions and a chloroform:isoamyl alcohol extraction were performed on the resulting solution, followed by precipitation with 0.1 volumes of 3 M sodium acetate (pH 5.0) and 2 volumes of ethanol. The precipitated DNA was treated as before, without repeating RNase treatment.

The purity and yield of DNA was assessed by spectrophotometric scan between wavelengths of 220 nm and 320 nm using a Beckman DU-64 spectrophotometer. An  $A_{260}$  reading of 1 corresponds to approximately  $50 \mu\text{g ml}^{-1}$  of ds DNA and an estimate of sample purity is provided by the ratio of the absorbance value at 260 nm to that at 280 nm, pure DNA having a value of 1.8. For plasmid DNA for nucleotide sequence determination only, samples were further purified using GeneClean II (BIO 101 Inc.) according to the manufacturer's instructions.

#### **2.2.10 Horizontal gel electrophoresis of DNA**

DNA samples were analysed by electrophoresis through gels prepared from agarose (ultra-pure) at concentrations between 0.7 and 1.0 % (w/v) in TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA, adjusted to pH 8.2 with glacial acetic acid). Samples were loaded with 0.2 volumes of TAE loading buffer (5 x TAE, 15 % [w/v] Ficoll 400, 0.25 M EDTA, 0.04 % [w/v] bromophenol blue and 0.04 % [w/v] xylene cyanol FF). Ethidium bromide was either included in the gel mix to a



final concentration of 0.5  $\mu\text{g ml}^{-1}$ , or the gel was stained after electrophoresis by incubation in a solution of that concentration for 30 min. The DNA was visualised using a short wavelength trans-illuminator (Hybaid). DNA size markers (1 kbp ladder) were purchased from Boehringer Mannheim.

#### **2.2.11 Restriction endonuclease analysis of DNA samples**

Digestions were carried out according to the manufacturers' recommended conditions, using buffers supplied by Boehringer Mannheim.

#### **2.2.12 Nucleotide sequence determination of ds plasmid DNA**

Plasmid DNA was isolated from *E. coli* as described in section 2.2.9. Plasmid DNA (4  $\mu\text{g}$  in a volume of 9  $\mu\text{l}$ ) was denatured by addition of 1  $\mu\text{l}$  2 M NaOH and incubation at 37 °C for 15 min. Annealing of the primer to the template was achieved by addition of 1  $\mu\text{l}$  of either the M13 -40 or reverse primers at 10 mM concentration, followed by precipitation by addition of 3  $\mu\text{l}$  3 M potassium acetate pH 5.5 and 75  $\mu\text{l}$  ethanol. The annealed template and primer DNA was redissolved in 12  $\mu\text{l}$   $\text{ddH}_2\text{O}$  and 2  $\mu\text{l}$  annealing mix (Pharmacia T7) added. Sequencing reactions (labelling and termination) were then carried out on the template DNA with the Pharmacia T7 Sequencing kit according to the manufacturer's instructions.

The samples generated by the sequencing reactions were heated at 80 °C for 2 min and resolved by electrophoresis through a 6 % (w/v) acrylamide, 7 M urea, 1 x TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0) gel. Following electrophoresis, the gel was fixed in 10 % (v/v) methanol, 10 % (v/v) acetic acid and dried under vacuum at 80 °C prior to autoradiography.

#### **2.2.13 Autoradiography**

DuPont Cronex 4 or Kodak Biomax X-ray film was exposed to filters or gels in autoradiography cassettes at room temperature. Films were developed using an X-Omat developer.



### **2.2.14 Computer analysis**

Analysis of nucleotide sequence data was carried out using the University of Wisconsin Genetics Computer Group package version 7 (GCG, Devereux *et al.*, 1984) through the VAX 8000 system at Edinburgh University.

## **2.3 Polymerase chain reaction (PCR) amplification of *Ms* and *Icl* cDNA fragments**

### **2.3.1 Reverse-transcription of post-germinative seedling RNA**

Total RNA from potato and *N. plumbaginifolia* seedlings at stage 3 was used in reverse-transcription reactions with Superscript II (Gibco BRL) according to the manufacturer's instructions. Approximately 10 µg of total RNA was used in each reverse-transcription reaction which was carried out in a total volume of 60 µl.

### **2.3.2 Design and synthesis of oligonucleotide primers for PCR**

PCR amplification reactions were carried out using the resulting cDNA with pairs of oligonucleotide primers designed by analysis of conserved regions in the cDNA sequences of other higher plants. The nucleotide sequences of these primers and their positions relative to the putative complete potato and *N. plumbaginifolia* *Ms* and *Icl* coding regions (assumed to be similar to those from cucumber) are shown in figures 2.2 and 2.3, respectively. Analysis of nucleotide sequences for design of primers was carried out using programmes of GCG version 7. PCR amplification of *Ms* cDNA fragments from both potato and *N. plumbaginifolia* was carried out using primers designed from a GCG 'Pileup' comparison of complete *Ms* cDNA sequences from *Brassica napus* (GenBank accession number J04468), cotton (X52305), pumpkin (X56948), cucumber (X15425) and castor bean (X52806). For PCR amplification of *Icl* cDNA fragments, two pairs of primers were used. For PCR amplification of the potato *Icl* cDNA fragment, primers used were a gift from A. Baker, University of Leeds. For PCR amplification of the *N. plumbaginifolia* *Icl*



***S. tuberosum* and *N. plumbaginifolia* *Ms* primers**

***Ms* primer 1:** 5' ATGGC**I**GAC/TTTTGAA/GGATGC 3'

***Ms* primer 2:** 3' CGTCC**I**GTA/GCTACCTTGIAC 5'

**Potato *Icl* primers**

***Icl* primer 1':** 5' AAG/AAAG/ATGT/CGGA/G/C/TCAT/CATG 3'

***Icl* primer 2':** 3' GGA/G/C/TT/AGA/G/C/TAAG/ATTA/GACCTAGGGG 5'

***N. plumbaginifolia* *Icl* primers**

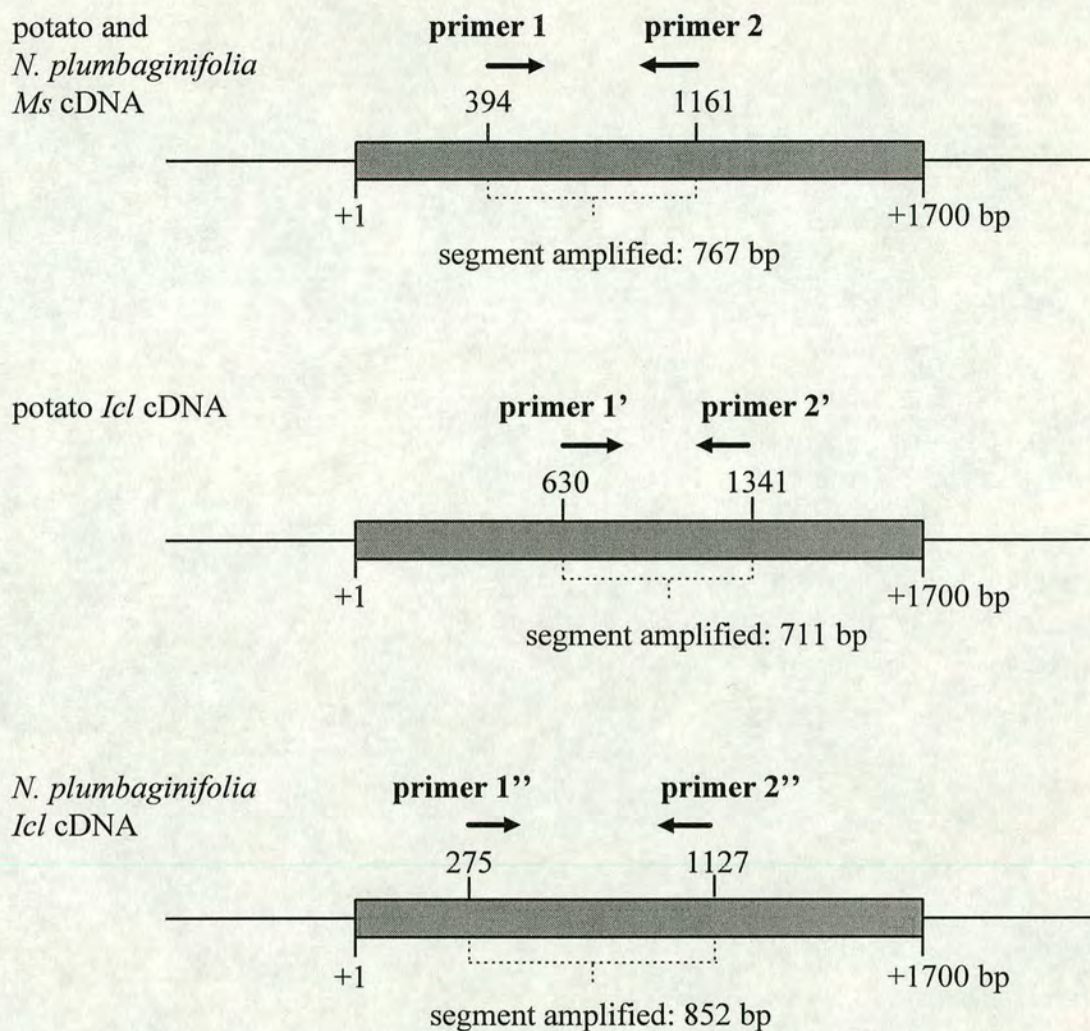
***Icl* primer 1'':** 5' CCATGATGGCTAAG/ACAT/CTTGG 3'

***Icl* primer 2'':** 3' GA**I**AAGACCCTG/AACCCTG/AAACGG 5'

**Figure 2.2: Primers for PCR amplification of *Ms* and *Icl* cDNA fragments**

Sequences of oligonucleotide primers used for polymerase chain reaction (PCR) amplification of cDNA segments. Primers 1, 1' and 1'': 'forward' primers, primers 2, 2' and 2'': 'reverse' primers, with respect to the putative coding sequences. Redundant positions represented either by partial synthesis or by inclusion of inosine are marked in bold, with backslashes separating the possible nucleotides at that position, or **I** for inosine. Primers for amplification of potato *Icl* were a gift from A. Baker, University of Leeds.





**Figure 2.3: PCR amplification of *Ms* and *Icl* cDNA fragments**

Positions of PCR primers with respect to the putative coding regions (shaded boxes) of *Ms* and *Icl* cDNAs. Primers 1 and 2, 1' and 2', 1'' and 2'' represent the different respective sets of primers (refer to fig. 2.2). Length of the putative coding regions (numbers indicate base pairs of coding region) is approximate. It was assumed that the cDNA sequences from potato and *N. plumbaginifolia* would be sufficiently similar to those used to design primers that segments of cDNA of the sizes denoted in this figure would be amplified by PCR.



cDNA fragment, design of oligonucleotide primers was based on the result of a 'Pileup' comparison of *Icl* cDNA sequences from cotton (GenBank accession number X52136), castor bean (M17145) and *Arabidopsis thaliana* (M83534).

In each case, two regions showing a high degree of homology at the nucleotide level identified by 'Pileup' were chosen to design pairs of primers. Nucleotide positions showing complete conservation in the organisms chosen for comparison were left unchanged in the respective oligonucleotide. Positions showing variation at the third nucleotide in a codon were occupied by an inosine residue. Positions other than codon third base showing variation up to two variant bases were represented in the oligonucleotide by synthesis with a mixture of the two nucleotides in equal proportions. Oligonucleotides were synthesised at the Oswel DNA service, University of Edinburgh.

### **2.3.3 PCR amplification reactions**

PCR amplification reactions were carried out with 10 µl of cDNA (section 2.3.1) using either Thermalase (IBI Ltd.) or *Taq* polymerase (Boehringer Mannheim) in the presence of the manufacturers' recommended buffers, 200 µM dNTPs and 0.25 µM of each species of oligonucleotide in mixtures representing redundant primers (except for amplification of the potato *Icl* cDNA fragment for which both primers were used at an overall concentration of 0.5 µM), in a total volume of 50 µl using a Hybaid thermal cycler. Evaporation from the reaction mixtures was reduced by addition of a layer of mineral oil. All four cDNA fragments were amplified by the following temperature cycles:

- i) 94 °C for 2 min (1 cycle)
- ii) 94 °C for 30 sec, 50 °C for 2 min, 70 °C for 1 min 30 sec (40 cycles. For the potato *Icl* cDNA fragment amplification reaction, this phase was extended to 60 cycles)
- iii) 70 °C for 5 min (1 cycle)
- iv) 30 °C (1 cycle).

Products of PCR amplifications were analysed by agarose gel electrophoresis. Fragments of sizes corresponding to those expected were purified from gels using



Geneclean II (BIO 101 Inc.) according to the manufacturer's instructions. In the case of the potato putative *Ms* cDNA fragment, the purified fragment was reamplified in a reaction identical to the initial PCR amplification and the resulting product purified.

## **2.4 Molecular cloning of DNA fragments**

### **2.4.1 Transformation of *E. coli***

All strains of *E. coli* were transformed with plasmid DNA by the following method (Chung *et al.*, 1989):

a fresh overnight culture of the strain to be transformed was diluted 1:100 [v/v] with LB broth and incubated at 37 °C with shaking until an  $A_{600}$  of between 0.3 and 0.4 was reached, after which the culture was chilled on ice for 10 min. 10 ml aliquots were centrifuged at approximately 8000 g for 10 min at 4 °C, the supernatant removed and each cell pellet resuspended in 1 ml of 1 x TSS (1 % [w/v] Tryptone [Difco], 0.5 % [w/v] Yeast extract [Difco], 1 % [w/v] NaCl, 20 mM  $MgSO_4$ , 20 mM PIPES [1,4-piperazinediethanesulfonic acid, pH 6.6], 10 % [w/v] polyethylene glycol  $M_r$  3,350, 5 % [v/v] freshly added DMSO) and stored on ice. Approximately 100 ng of plasmid DNA in a volume of 10  $\mu$ l or less was added to 200  $\mu$ l aliquots of the cells, mixed and incubated on ice for 30 min, after which 0.9 ml of LB containing 10 mM glucose was added to each aliquot. These were then incubated at 37 °C for a minimum of 1 h, and 100  $\mu$ l aliquots of the cells spread onto LB agar for selection of transformant cells.

### **2.4.2 Transformation of *A. tumefaciens***

*A. tumefaciens* strain LBA4404 was transformed with pBI121-based plasmids by freeze-thaw direct transformation (Gelvin *et al.*, 1988).



### **2.4.3 Preparation of plasmid vectors for ligation to DNA fragments**

Plasmid vectors into which DNA fragments were to be inserted were digested with the appropriate restriction enzyme(s) and the linearised vector DNA then purified by addition of, and mixing with, an equal volume of phenol. Traces of phenol in the resulting aqueous phase were then removed by addition of an equal volume of chloroform:isoamyl alcohol (24:1 v/v). DNA was precipitated by addition of 0.1 volumes of 3 M sodium acetate (pH 5.5) and 2 volumes of ethanol, recovered by centrifugation at 12000 g for 10 min and redissolved in  $\text{sddH}_2\text{O}$ . The yield of DNA was estimated by electrophoresis of an aliquot through 0.8 % (w/v) agarose.

In the case of the potato *Ms* cDNA fragment, pUC19 used for subcloning was treated with calf intestinal phosphatase (CIP) to prevent religation of the blunt ends which resulted from digestion of this plasmid with *Sma*I. 0.1 volumes of 10 x CIP dephosphorylation buffer (10 mM  $\text{ZnCl}_2$ , 10 mM  $\text{MgCl}_2$ , 100 mM Tris-HCl pH 8.3) and 1 U CIP were added to 2  $\mu\text{g}$  digested pUC19 and the mixture incubated at 37 °C for 15 min then 56 °C for 15 min, 1 U CIP added to the mixture and the incubations repeated. The CIP-treated vector was purified by electrophoresis through 0.8 % (w/v) agarose followed by treatment with GeneClean II (Bio 101 inc.).

The  $\beta$ -glucuronidase gene contained within pBI121 was excised, before this vector was used as the final recipient of the cDNA fragments, by digestion with *Xba*I and *Sac*I, or *Sma*I and *Sac*I (section 2.4.4), and the digestion products separated by agarose gel electrophoresis after which the larger fragment (containing the CaMV 35S promoter) was purified from the gel by GeneClean II (Bio 101 inc.).

### **2.4.4 Ligation of fragment DNA to vector DNA**

Fragment and vector DNA were ligated by mixing together 100 ng of linearised vector DNA with either a 10 fold molar excess of fragment (ligations involving DNA with blunt ends) or a 3 fold molar excess of fragment (ligations involving DNA with cohesive ends),  $\text{sddH}_2\text{O}$  to a volume of 17  $\mu\text{l}$ , 2  $\mu\text{l}$  10 x ligation buffer (200 mM Tris-HCl pH 7.6, 50 mM  $\text{MgCl}_2$ , 50 mM DTT, 5 mM ATP) and 4 Weiss U T4 DNA ligase. The reaction mix was incubated at either 37 °C for 4 h (ligations involving



DNA with blunt ends) or 16 °C for 18 h (ligations involving DNA with cohesive ends).

#### **2.4.5 Insertion of PCR amplification products into plasmid vectors**

Each of the four PCR amplification products were inserted into the polylinker of pBluescript II SK+ by one of two methods:

i) The potato putative *Ms* and *Icl* cDNA fragments were inserted by ligation to the blunt termini of pBluescript II SK+ resulting from digestion of the vector with *Sma*I, resulting in plasmids named pBlScPMS31 and pBlScPICL4, respectively. The ends of the PCR amplification products were rendered blunt by treatment with T4 DNA polymerase. Approximately 1 µg of fragment was used in a reaction with 2 U of T4 DNA polymerase in the presence of 5 mM MgCl<sub>2</sub> and 100 µM dNTPs at 12 °C for 20 min. The DNA was then purified by addition of, and mixing with, an equal volume of phenol. Traces of phenol in the resulting aqueous phase were then removed by addition of an equal volume of chloroform:isoamyl alcohol (24:1 v/v). The DNA was precipitated by addition of 0.1 volumes of sodium acetate and 2 volumes of ethanol, recovered by centrifugation at 12000 g for 10 min and redissolved in ddH<sub>2</sub>O. Ligations were carried out under conditions suitable for DNA with blunt ends (section 2.4.2).

ii) The *N. plumbaginifolia* putative *Ms* and *Icl* cDNA fragments were inserted by ligation to linearised pBluescript II SK+ which had had a single dTTP residue added to the 3' termini at each end of the DNA duplex ('T-tailed' vector, Marchuk *et al.*, 1991). This should aid annealing of the vector ends to the ends of PCR amplification products to which Thermalase and *Taq* polymerase have added a single dATP residue on completion of each cycle of amplification. For ligation with the *N. plumbaginifolia* putative *Ms* cDNA fragment, pBluescript II SK+ was 'T-tailed' after linearisation by digestion with *Sma*I. The resulting plasmid was named pBlScTMS6. For ligation with the *N. plumbaginifolia* putative *Icl* cDNA fragment, pBluescript II SK+ was 'T-tailed' after linearisation with *Eco*RV. The resulting plasmid was named pBlScTICL10. Both forms of 'T-tailed' pBluescript II SK+ were provided by I.



Oliver, University of Edinburgh. Ligations were carried out under conditions suitable for DNA with cohesive ends.

#### 2.4.6 Subcloning of cDNA fragments in pUC19 and pBI121

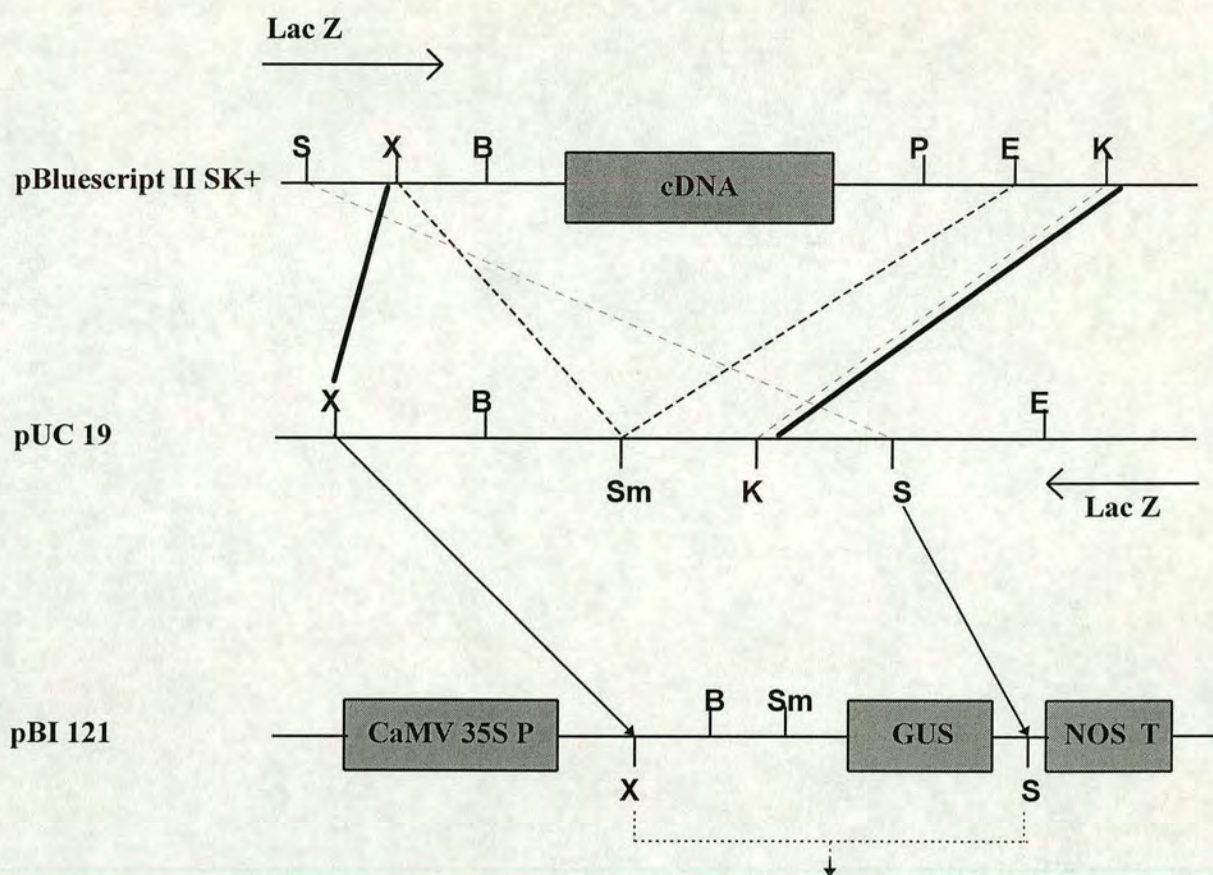
Subcloning schemes used to transfer the four cDNA fragments from pBluescript II SK+ to pBI121 in the antisense orientation with respect to the CaMV 35S promoter are shown in fig. 2.4. The host strain in which plasmids resulting from subcloning steps were harboured was XL1-Blue. These steps were as follow:

i) Potato *Ms* cDNA fragment: the cDNA fragment was excised from pBluescript II SK+ by digestion with *Xba*I and *Eco*RI, the termini rendered blunt-ended by treatment with T4 DNA polymerase and ligated to the blunt ends of CIP-treated pUC19 resulting from digestion of this plasmid with *Sma*I. The resulting plasmid was digested with *Xba*I and *Sac*I and the cDNA fragment excised was ligated to pBI121 which had been digested with *Xba*I and *Sac*I. The resulting plasmid was named pBIPMS1.

ii) Potato *Icl* cDNA fragment: the cDNA fragment was excised from pBluescript II SK+ by digestion with *Kpn*I and *Sac*I and ligated to pUC19 which had also been digested with *Kpn*I and *Sac*I. The resulting plasmid was digested with *Sma*I (recognition site within the PCR primer sequence) and *Sac*I and the cDNA fragment excised was ligated to pBI121 which had also been digested with *Sma*I and *Sac*I. The resulting plasmid was called pBIPICL1.

iii) *N. plumbaginifolia* *Ms* and *Icl* cDNA fragments: the cDNA fragments were excised from pBluescript II SK+ by digestion with *Kpn*I and *Xba*I and ligated to pUC19 which had also been digested with *Kpn*I and *Xba*I. The resulting plasmids were digested with *Xba*I and *Sac*I and the cDNA fragments excised were ligated to pBI121 which had also been digested with *Xba*I and *Sac*I. The resulting plasmids were named pBITMS1 and pBITICL1, respectively.





**Figure 2.4: Construction of antisense *Ms* and *Icl* cDNA transformation vectors**

Only the multicloning site of each vector is shown. Dashed lines represent scheme used for potato *Ms* cDNA fragment (blunt-ended insertion), dotted lines for scheme used for potato *Icl* cDNA fragment, bold lines for scheme used for *N. plumbaginifolia* *Ms* and *Icl* cDNA fragments. Restriction endonuclease recognition sites are represented by single letters: S: *Sac*I, X: *Xba*I, B: *Bam*HI, P: *Pst*I, E: *Eco*RI, K: *Kpn*I, Sm: *Sma*I. All *Ms* and *Icl* cDNA fragments were inserted into pBI121 as *Xba*I - *Sac*I fragments. **CaMV 35S P**: Cauliflower mosaic virus 35S promoter. **GUS**:  $\beta$ -glucuronidase reporter gene. This segment of pBI121 was removed prior to insertion of fragments. **NOS T**: Nopaline synthase terminator. **Lac Z**: *E. coli lacZ* gene, arrow indicates direction of transcription.



#### 2.4.7 Verification of *pBI121*-derived plasmids following subcloning

The following methods were used to verify that no rearrangements of the cDNA fragments had occurred within the *pBI121*-derived plasmids prior to transformation of plant tissue:

i) Potato *Ms* cDNA fragment: the cDNA fragment was excised from *pBIPMS1* by digestion with *Xba*I and *Sac*I and ligated to *pBluescript* II SK+ which had also been digested with *Xba*I and *Sac*I. The nucleotide sequence of the cDNA fragment was then redetermined from the resulting plasmid. LBA4404 was then transformed with *pBIPMS1*. Plasmid DNA was extracted from the resulting strain, named LBAPMS1, and immediately used to transform XL1-Blue from which plasmid DNA was extracted and the size and orientation of the cDNA fragment verified by digestion with a panel of restriction endonucleases.

ii) Potato *Icl* cDNA fragment: the procedure was as for the potato *Ms* cDNA fragment but the cDNA fragment was excised with *Sma*I and *Sac*I, ligated to *Sma*I and *Sac*I-digested *pBluescript* II SK+ and the final LBA4404-derived strain was named LBAPICL3.

iii) *N. plumbaginifolia* *Ms* cDNA fragment: the procedure was as for the potato *Ms* cDNA fragment and the final strain was named LBATMS2.

iv) *N. plumbaginifolia* *Icl* cDNA fragment: LBA4404 was transformed with *pBITICL1* before nucleotide sequence determination, resulting in a strain named LBATICL2. Plasmid DNA was extracted from this strain and used to transform XL1-Blue, from which *pBITICL1* was re-extracted and the cDNA fragment excised and religated to *pBluescript* II SK+ as for the potato *Ms* and *N. plumbaginifolia* *Icl* cDNA fragments, prior to nucleotide sequence determination and restriction endonuclease analysis.



2.5 Plant transformation

2.5.1 Media

Medium:	Potato:	<i>N. plumbaginifolia</i> :
MS	1 x M & S salts (4.71 g l <sup>-1</sup> ), 30 g l <sup>-1</sup> sucrose, pH 5.8.	1 x M & S, 10 g l <sup>-1</sup> sucrose, 2 µg ml <sup>-1</sup> kinetin, 0.2 µg ml <sup>-1</sup> NAA, pH 5.8.
Callus induction	As MS, with 2 mg l <sup>-1</sup> zeatin riboside, 0.2 mg l <sup>-1</sup> NAA, 0.02 mg l <sup>-1</sup> GA <sub>3</sub> .	—
Shooting	As callus induction, but with 0.02 mg l <sup>-1</sup> NAA.	As MS.
Expansion	—	0.5 x M & S, 5 g l <sup>-1</sup> sucrose, pH 5.8.
Rooting	As MS, with 10 g l <sup>-1</sup> sucrose.	As expansion, without sucrose.
Nodal	0.5 x M & S, 15 g l <sup>-1</sup> sucrose, 0.2 µg ml <sup>-1</sup> kinetin, pH 5.8.	—

NAA:  $\alpha$  naphthaleneacetic acid. GA<sub>3</sub>: gibberellic acid. Media were either in liquid (no agar) or solid form by addition of 0.8 % (w/v) bacto-agar. Unless otherwise stated, media were in solid form. The appropriate antibiotics (carbenicillin to stop growth of *A. tumefaciens* and kanamycin to select for transformed tissue) were added to potato transformation media at the following concentrations: 500 µg ml<sup>-1</sup> carbenicillin, 50 µg ml<sup>-1</sup> kanamycin. For *N. plumbaginifolia* media, these were: shooting, 500 µg ml<sup>-1</sup> carbenicillin, 200 µg ml<sup>-1</sup> kanamycin; expanding, 250 µg ml<sup>-1</sup> carbenicillin, 200 µg ml<sup>-1</sup> kanamycin; rooting, 100 µg ml<sup>-1</sup> each of carbenicillin and kanamycin. Regenerated plants were grown in sterile conditions in petri dishes, glass jars or polypropylene tubes until transfer to soil.



### 2.5.2 Transformation of potato tissue

*A. tumefaciens* cultures were grown for 48 h with the appropriate antibiotics and 3 ml aliquots were washed by centrifugation and resuspension in LB broth without antibiotics. Segments of internode 4 to 5 mm in length (approximately 50 segments for each transformation) were removed under sterile conditions from 3 to 5 week old plantlets derived from sterile nodal cultures maintained on nodal medium. These were placed in 20 ml of liquid MS medium to which the 3 ml of the appropriate strain of *A. tumefaciens* had been added and the mixture gently agitated for 15 min in darkness at room temperature. The stem pieces were then placed flat onto callus induction medium without antibiotics and incubated for 48 h in darkness at 25 °C. They were then transferred to callus induction medium containing carbenicillin and incubated in a constant irradiance of  $120 \mu\text{mol m}^{-2} \text{s}^{-1}$  at 25 °C for 4 days, then transferred to callus induction medium containing carbenicillin and kanamycin. The segments were subcultured (placed on fresh medium) every 2 weeks until callus began to emerge beyond approximately 2 mm from the cut ends of the stem segments, at which time they were transferred to shooting medium containing carbenicillin and kanamycin and subcultured every 2 weeks until shoots (approximately 1 cm long) were regenerated. Shoots were excised at the stem and transferred to rooting medium containing carbenicillin and kanamycin and allowed to grow. After 8 weeks, plants with roots were transferred to soil.

### 2.5.3 Transformation of *N. plumbaginifolia* tissue

Expanded leaves were surface sterilised with 70 % (v/v) ethanol for 30 sec followed by 10 % (v/v) sodium hypochlorite for 15 min and washed in  $\text{ddH}_2\text{O}$  six times. Under sterile conditions, leaf discs of 1 cm diameter were cut, avoiding the mid-rib and major veins, and placed in 100 ml of liquid MS medium containing the appropriate *A. tumefaciens* strain (prepared as for potato transformations) and the mixture gently agitated for 15 min. Discs were then transferred flat (cuticle uppermost) to solid shooting medium and incubated under constant irradiance of  $120 \mu\text{mol m}^{-2} \text{s}^{-1}$  at 25 °C for 48 h. The discs were then transferred to shooting medium



containing carbenicillin and kanamycin and culture continued under the same conditions with transfer to fresh medium every 3 weeks until shoots were regenerated. Shoots were transferred onto plates of expanding medium containing carbenicillin and kanamycin. After 4 weeks, those which continued to expand were transferred to rooting medium containing carbenicillin and kanamycin. Once shoots had rooted, plantlets were transferred to soil.

## **2.6 MS activity assays**

### **2.6.1 *Crude protein extraction and MS assays***

Seedlings at stage 3 of post-germinative growth were selected and weighed. For every g of seedling material, 1 ml of homogenisation buffer (50 mM Tris-HCl, pH 8.5, 50 mM potassium acetate pH 5.5, 5 mM magnesium acetate, 0.2 mM DTT) was added. The seedlings were homogenised with a micro-pestle and the supernatant separated from the debris by centrifugation at 12000 g for 30 min at 4 °C. Assays (based on the method of Cooper & Beevers, 1969b) were carried out by adding 0.1 ml of DTNB solution (100mM Tris-HCl pH 8.0, 1 mM dithiobisnitrobenzoic acid, 10 mM MgCl<sub>2</sub>), 0.1 ml homogenate and 0.1 ml of 0.2 mM acetyl-CoA (in 100 mM Tris-HCl pH 8.0) to 0.6 ml of 100 mM Tris-HCl pH 8.0. Absorbance was monitored at 412 nm for 2 min (until stable) and then 0.1 ml of 20 mM glyoxylate (sodium salt) in 100 mM Tris-HCl, pH 8.0 was added to the mixture to start the reaction. Absorbance at 412 nm was monitored for 5 min and the rate of reaction at 2 min after addition of glyoxylate measured.

### **2.6.2 *Total protein assays***

Total protein content of the samples was measured using the Bio-Rad Protein Assay system (Bio-Rad Laboratories) according to the manufacturer's instructions. These measurements were then used to standardise the MS activity readings.



### **3. PROJECT AIMS**



Although many biochemical aspects of the glyoxylate cycle are understood, little is known about its physiological significance. It is thought to fulfill an important requirement during several key developmental stages, but there is no evidence to suggest that the glyoxylate cycle is indispensable for these. It was the aim of this project to determine whether or not this is the case, by inhibition of MS and ICL synthesis in transgenic plants through the use of chimaeric antisense genes comprising fragments of *Ms* and *Icl* cDNAs in the antisense orientation with respect to the CaMV 35S promoter.

The effects of inhibiting the synthesis of MS or ICL may be important in several respects. These are as follow:

1) Interference with any developmental stage during which MS and ICL are known to be synthesised (see section 1.4). This includes post-germinative growth, embryogenesis, senescence and pollen formation. If the glyoxylate cycle is necessary for embryogenesis or post-germinative growth, it is anticipated that inhibition of MS or ICL synthesis would result in the production of inviable seed. Similarly, interference with pollen formation would result in infertile plants. Interference with lipid mobilisation during senescence could result in alterations in carbon partitioning.

Func.  
seed form

2) Control of the glyoxylate cycle. Any effects on MS synthesis caused by the introduction of an antisense ICL gene, and *vice versa*, may reveal aspects of the regulation of the glyoxylate cycle. Evidence suggests that through physical association of peroxisomal proteins, metabolite channeling may exist in peroxisomes (see section 1.2.5). If a degree of interdependence between glyoxysomal proteins does exist, inhibition of the synthesis of one protein might be expected to influence the activity or synthesis of others.

3) Mechanisms of antisense inhibition. Inhibition of *Ms* and *Icl* gene expression with antisense genes would provide an opportunity to study how the relationship between mRNA and protein levels is affected. This would contribute to general understanding of the mechanisms of antisense inhibition.



Two plant species were selected for antisense inhibition of MS and ICL synthesis, potato and *N. plumbaginifolia*. Transformation of *N. plumbaginifolia* can be achieved with relative ease and rapidity, and is amenable to genetic analysis. However, it was hypothesised that inhibition of MS or ICL synthesis would prevent sexual reproduction due to possible effects on post-germinative growth, pollen development or embryogenesis. This would preclude propagation of *N. plumbaginifolia* for further analysis. This potential problem is circumvented by the use of potato, which can be propagated vegetatively and has no requirement for sexual reproduction. However, effects on sexual reproduction could be subsequently studied in plants found to have reduced MS or ICL activity. Tuber formation also provides an opportunity to study effects on carbon partitioning in future studies.

The initial focus of this project was therefore the generation in parallel of transgenic *N. plumbaginifolia* and potato plants carrying chimaeric antisense *Ms* or *Icl* genes. Two sets of transformants were to be generated for each species, one set carrying a chimaeric antisense *Ms* gene comprising *Ms* cDNA from the respective species and the other set carrying a chimaeric antisense *Icl* gene comprising *Icl* cDNA from the respective species. Following generation of transgenic plants, the effect of the antisense gene on enzyme activity and mRNA steady-state levels was to be assessed, together with identification of possible consequences for development. This was to include effects on tuber formation and seed production or viability.



## **4. RESULTS**



## 4.1 Project strategy

The project described in this section consisted of three main parts: i) the isolation of *Ms* and *Icl* cDNA fragments from potato and *N. plumbaginifolia* by PCR amplification, cloning of these and construction of chimaeric antisense genes, ii) transformation of potato and *N. plumbaginifolia* tissue with the chimaeric antisense genes and regeneration of transformed plants, iii) analysis of transformants.

## 4.2 Construction of chimaeric antisense genes

### 4.2.1 Isolation of *Ms* and *Icl* cDNA fragments

*Ms* and *Icl* cDNAs from potato and *N. plumbaginifolia* were not available prior to commencement of this project. The first step of the project was therefore to obtain these, and this was achieved by PCR amplification. Total RNA was extracted from potato and *N. plumbaginifolia* seedlings at stage 3 of post-germinative growth (see figure 2.1), a stage of development in cucumber when the *Ms* and *Icl* transcripts are known to be abundant. The abundance of these transcripts at stage 3 in *N. plumbaginifolia* was confirmed by northern hybridisation (see section 4.6.1.1 and figure 4.3). cDNA was synthesised from stage 3 total RNA by reverse-transcription and used in PCR amplifications using pairs of primers designed by analysis of conserved regions of sequences in other organisms. For both sets of *Ms* primers and *N. plumbaginifolia* *Icl* primers, design was based on analysis of nucleotide sequences from higher plants. For potato *Icl*, primers used were a gift from A. Baker, University of Leeds, and had been designed from analysis of polypeptide sequences in microorganisms. These primers are shown in figure 2.2.

The positions of each pair of primers relative to the potato and *N. plumbaginifolia* putative *Ms* and *Icl* coding regions, and hence predicted sizes of the amplification products, are shown in figure 2.3. The complete coding regions of potato and *N. plumbaginifolia* *Ms* and *Icl* were assumed to be the same size as those for other plants. Based on this, the fragments generated were expected to include a



central segment of between approximately 40 % and 50 % of the putative coding regions. In each case, only single amplification products of the expected sizes were generated under optimal conditions.

All amplification products were cloned by ligation to pBluescript II SK+ and correct insertion verified by restriction endonuclease analysis. Partial nucleotide sequences of the putative cDNA fragments were obtained from each end and compared with other *Ms* and *Icl* sequences available on the GenEMBL database. The partial nucleotide sequences and their similarities to *Ms* and *Icl* in other organisms are shown in figures 4.1 and 4.2, and table 4.1, respectively. The high degree of homology at the nucleotide level between the amplification products and the *Ms* and *Icl* sequences with which they are compared (particularly where the comparisons are with higher plant sequences) suggested strongly that they were genuine *Ms* and *Icl* cDNA fragments. The plasmids containing the fragments with the sequences presented in figures 4.1 and 4.2 (pBIScPMS31, pBIScPICL4, pBIScTMS6 and pBIScTICL10, respectively) were therefore selected for further work.

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AS.

#### 4.2.2 Construction of plasmids for plant transformation

The cDNA fragments were excised from the pBluescript-based plasmids and initially subcloned in pUC19. This step was included in order to provide a means for ensuring that the cDNA fragments could subsequently be ligated to the plant transformation vector pBI121 in a predetermined orientation with respect to the promoter. All fragments were successfully cloned in pBI121 by the subcloning scheme represented in figure 2.4. The orientation and sizes of the fragments at each successive step were verified by restriction endonuclease analysis and, at the final stage prior to plant transformation, nucleotide sequence determination of all junction sites and the extremities of the cDNA fragments. No rearrangements to the cDNA fragments were detected by the techniques employed and the pBI121-based plasmids carrying the potato *Ms* cDNA fragment (pBIPMS1), the potato *Icl* fragment (pBIPICL1), the



**Figure 4.1: Sequences of PCR-amplified putative *Ms* and *Icl* cDNA segments from potato**

Positions of PCR primers marked in bold type.

A) Partial sequence corresponding to **5' end of PCR-amplified potato *Ms* cDNA** sequence:

```
5' 1  ATGGCGGATT TTGAGGATGC ACTGTCACCA AGCTGGGAGA ATTTAATGAG .
      51  AGGCCATGTA AATTTGAGGG ATGCAGTGAA TAGAACAATA ACATTCCATG
      101  ATCAAGCCAG AAACAAAGTG TATAAACTGA ATGATCAGAC AGCCAAGTTG
      151  TTTGTGCGCC CAAGAGGGTG GCATCTGCCA GAAGCTCACA TCTTCATTGA
      201  TGGTGAGCCT GCAACAGGTT GCCTTGTCGA CTTCGGCCTC TACTTCTTCC
      251  ACAGCTATGC CAACTTCCCG CAAGCCCAAG GACAAGGATT TGACCCTATT
      301  TTCTATCTTC CCAAGATGGA ACATTCTAGG G 331 3'
```

B) Partial sequence corresponding to **3' end of PCR-amplified potato *Ms* cDNA** sequence:

```
5' 1  GAACACTCCA GCAGTGTTTC AGATGAATGA AATCTTATAT GAACTGAGGT
      51  CCATTTCAGTT GGCCTCAACT GTGGTAGATT GGATTACATT TTCAGCTACG
      101  TCAAGACTTT CCAGGGTCAT CCCGATCGAT TACTCCCTGA TAGGGTTCAA
      151  GTTGGCATGG CTCAACACTT TATGAGGAGT TACTCTGACT TGCTCATCCA
      201  TACCTGTCAT AAGCGCGATG TCCATGCTAT GGGAGGCATG GCAGCTCAAA
      251  TTCCAATCAG AGATGATCCA GCAGCTAACG AGGCAGCATT GGAAC TAGTA
      301  AGGAAGGATA AGCTGAGAGA AGTGAAGGCA GGCCACGATG GAACCTG 347
                                           3'
```



C) Partial sequence corresponding to **5' end of PCR-amplified potato *Icl* cDNA** sequence:

```
5' 1  AGAAGTGC GG GCACATGGCT GGTAAAGTGC TTGTTGCTGT AAGTGAACAT
    51  ATTAACAGGC TTGTGGCTGC AAGGTTGCAG TTTGATATCA TGGGGACTGA
    101  AACGGTCTCG TGGCTCGTAC TGATGCGGTT GCAGCCACAT TGATCCAACA
    151  TGTGATACAG GATCACAGTC 170 3'
```

D) Partial sequence corresponding to **3' end of PCR-amplified potato *Icl* cDNA** sequence:

```
5' 1  GAGACATCAA GTCCTGATAT GGTGGAATGC ACAAATTTT CTGAAGGAGT
    51  GAAGTCTCTA AAGCCTGAGC TGATGTTGGC TTACAATCTG TCTCCCACCT
    105  TCAACTGGAT CCCC 114 3'
```



**Figure 4.2: Sequences of PCR-amplified putative *Ms* and *Icl* cDNA segments from *N. plumbaginifolia***

Positions of PCR primers marked in bold type.

A) Partial sequence corresponding to **5' end of PCR-amplified *N. plumbaginifolia* *Ms* cDNA** sequence:

```
5' 1  ATGGCGGATT TTGAAGATGC ACTTTCACCA AGTTGGGAGA ATCTAATGAG
    51  AGGCCAAGTA AATTTGAGGG ATGCAGTGAA TGGAACAATA TCATTCCATG
    101  ATCAAGCCAG AAACAGAGTT TATAAACTGA ATGATCAGAC GGCTAAGCTT
    151  TTCGTTGCCG CCCCAAGAGG CTGGCATTG CCTGAAGCCT CACATCT 197
                                           3'
```

B) Partial sequence corresponding to **3' end of PCR-amplified *N. plumbaginifolia* *Ms* cDNA** sequence:

```
5' 1  CTGATAGAGT TCTTGTGGC ATGTCTCAAC ACTTTATGAG AAGTTACTCT
    51  GACTTGCTCA TCCACACCTG TCATAAGCGT GCGTCCATG CAATGGGAGG
    101  CATGGCAGCT CAGATTCCAA TCAGAGATGA TCCAGCAGCA AATGAGGCAG
    151  CATTGGAACT AGTAAGGAAA GACAAGCTAA GAGAAGTGAA GGCAGGCCAT
    201  GATGGAACCT G 211 3'
```

C) Partial sequence corresponding to **5' end of PCR-amplified *N. plumbaginifolia* *Icl* cDNA** sequence:

```
5' 1  CCATGATGGC TAAACACTTG GACTCAATCT ATGTTTCTGG TTGGCAGTGT
    51  TCCTCAACTC ACACCACATC CAATGAACCA GGTCCTGATC TTGCTGATTA
    101  CCCTTATGAC ACAGTTCCAA ACAAAGTGGA ACATCTGTTT ATGGCTCAAC
    151  AGTATCATGA TAGGAAACAA AGGGAAGCAA GAATGAGA 188 3'
```



D) Partial sequence corresponding to **3' end of PCR-amplified *N. plumbaginifolia* *Icl* cDNA** sequence:

```
5' 1  TTCAAGCCTG GAGGATAAAT GGCTAGCAAT GGCTGAACTC AAGACATTTT
    51  CTCAATGTGT GATTGATGCA ATCAAGAAAA TGAACGTTAC GGAGTCCGAA
    101  AAGCAAAGGA GATTGAACGA GTGGATGGCT CATTCAAGTT TTGAAAAATG
    151  TCTTTCACAT GAGCAAGCTC GTGAAATTAC TGAAAGACTT GGACTTCCAA
    201  ATCTCTTCTG GGACTGGGAT TTGCC 226 3'
```



**Table 4.1: Homology between sequences of PCR products and *Ms* or *Icl* in other organisms.** Comparisons at the nucleotide level between sequences obtained for the 5' segments of the PCR products obtained using *Ms* and *Icl* primers (figure 2.2), and nucleotide sequences available on the GenEMBL database. Comparisons were carried out using the GCG 'Gap' program. **A)** comparisons between **potato** putative *Ms* cDNA sequence, **5' segment**, and *Ms* coding sequences from other organisms. **B)** comparisons between **potato** putative *Icl* cDNA sequence, **5' segment**, and *Icl* coding sequences from other organisms. **C)** comparisons between *N. plumbaginifolia* putative *Ms* cDNA sequence, **5' segment**, and *Ms* coding sequences from other organisms. **D)** comparisons between *N. plumbaginifolia* putative *Icl* cDNA sequence, **5' segment**, and *Icl* coding sequences from other organisms.

**Potato cDNA comparisons**

<b>A) <i>Ms</i></b>		<b>B) <i>Icl</i></b>	
<b>organism</b>	<b>% identity</b>	<b>organism</b>	<b>% identity</b>
<i>E. coli</i>	57.1	<i>E. coli</i>	57.1
<i>N. crassa</i>	52.8	<i>S. cerevisiae</i>	62.9
cucumber	78.2	cotton	75.3
pumpkin	79.2	castor bean	70.0
cotton	75.2	<i>A. thaliana</i>	67.1
castor bean	77.9		
<i>B. napus</i>	74.3		
<i>N. plumbaginifolia</i>	87.5		

***N. plumbaginifolia* cDNA comparisons**

<b>C) <i>Ms</i></b>		<b>D) <i>Icl</i></b>	
<b>organism</b>	<b>% identity</b>	<b>organism</b>	<b>% identity</b>
<i>E. coli</i>	47.4	<i>E. coli</i>	39.4
<i>N. crassa</i>	47.7	<i>S. cerevisiae</i>	74.5
cucumber	79.2	cotton	83.5
pumpkin	77.6	castor bean	85.6
cotton	72.5	<i>A. thaliana</i>	81.9
castor bean	76.2		
<i>B. napus</i>	70.5		
potato	87.5		



*N. plumbaginifolia* *Ms* fragment (pBITMS1) and the *N. plumbaginifolia* *Icl* fragment (pBITICL1) were transferred into the *A. tumefaciens* strain LBA4404.

### 4.3 Potato transformation and regeneration of transformants

Potato internodal sections were transformed by infection with *A. tumefaciens* strains carrying the plasmids pBIPMS1 or pBIPICL1. A total of 15 plantlets were regenerated from the transformation with pBIPMS1 (hereafter referred to as PMS plants) and 19 from the transformation with pBIPICL1 (hereafter referred to as PICL plants). Control plants were regenerated from two types of transformation, infection of tissue with the untransformed *A. tumefaciens* strain LBA4404 (which should give rise to wild-type plants), and infection of tissue with LBA4404 carrying the unaltered vector pBI121 (which contains the *gus* gene under the control of the CaMV 35S promoter). Resistance to kanamycin is conferred by the *nptII* gene carried on pBI121. The ability of PMS, PICL and pBI121 (control) tissue to successfully undergo shooting and rooting in medium containing kanamycin was the basis for identifying and propagating transformants. Each original transformant was used to generate two plants, one of which was maintained as a sterile *in vitro* culture and the other allowed to grow to maturity in soil under greenhouse conditions. No transformants appeared to show any differences in superficial growth characteristics compared to control plants. The tubers from each plant grown to maturity were harvested, counted and the total of each plant weighed. These data are listed in table 4.2. Although there was some indication of potentially significant differences between transformants in terms of tuber numbers, low yields or degree of sprouting, these data were not analysed further as seed from the *N. plumbaginifolia* transformants was found to be viable (see section 4.4.3). All tubers were subsequently propagated by H. Davies at the Scottish Crop Research Institute, Invergowrie, for potential future analysis.



**Table 4.2: Production of PICL and PMS tubers.**

**LBA4404 A1:** control plant generated from transformation with untransformed strain LBA4404. **pBI121 A1:** control plant generated from transformation with LBA4404 carrying the plasmid pBI121. Weight figures are given to one decimal place. **Tuber sprouting:** degree of tuber sprouting, arbitrary scale relative to control plants, after 11 months at 4 °C.

<b>T<sub>0</sub> plant:</b>	<b>Number of tubers:</b>	<b>Total weight (g):</b>	<b>Mean weight (g):</b>	<b>Tuber sprouting:</b>
LBA4404 A1	61	154.9	2.5	10
pBI121 A1	24	245.3	10.2	10
PICL A1	78	191.0	2.5	4
PICL A2	39	195.7	5.0	8
PICL A3	24	147.3	6.1	9
PICL A4	27	109.1	4.0	10
PICL A5	8	16.9	2.1	3
PICL A6	11	93.6	8.5	10
PICL B1	17	137.6	8.1	10
PICL B2	37	116.9	3.2	8
PICL B3	46	95.4	2.1	10
PICL B4	11	155.2	14.1	10
PICL B6	36	107.4	3.0	5
PICL C1	19	99.5	5.2	5
PICL C2	40	124.1	3.1	4
PICL C3	14	61.3	4.4	10
PICL C7	20	189.8	9.5	6
PICL C8	20	217.9	10.9	10
PICL C9	1	18.3	18.3	0
PICL C10	23	119.5	5.2	1
PICL C12	22	197.6	9.0	10
PMS A1	74	172.0	2.3	1
PMS A2	14	52.4	3.7	2
PMS A3	37	115.2	3.1	10
PMS A4	25	197.4	7.9	10
PMS B1	40	243.6	6.1	10
PMS B3	38	112.9	3.0	10
PMS B5	7	100.6	14.4	0



Table 4.2, continued.

T <sub>0</sub> plant:	Number of tubers:	Total weight (g):	Mean weight (g):	Tuber sprouting:
PMS B7	1	8.7	8.7	0
PMS B8	12	59.9	5.0	2
PMS B11	22	136.5	6.2	6
PMS B12	26	213.2	8.2	8
PMS C1	28	263.2	9.4	8
PMS C2	16	239.3	15.0	10
PMS C3	24	176.5	7.4	10
PMS C4	15	184.0	12.3	6



#### **4.4 *N. plumbaginifolia* transformation and regeneration of transformants**

*N. plumbaginifolia* leaf discs were transformed by infection with *A. tumefaciens* strains carrying the plasmids pBITMS1 or pBITICL1. A total of 59 plants were regenerated from the transformation with pBITMS1 (hereafter referred to as TMS plants) and 23 from the transformation with pBITICL1 (hereafter referred to as TICL plants). Control plants of the same type as described in section 4.3 were generated from *N. plumbaginifolia* tissue. As with the potato transformations, the ability of TMS, TICL and pBI121 (control) tissue to successfully undergo shooting and rooting in medium containing kanamycin was the basis for identifying and propagating transformants. After successful rooting *in vitro*, plants were transferred to soil and grown to maturity under greenhouse conditions.

##### **4.4.1 *The Low Seed Producing (LSP) phenotype***

Plants were allowed to flower and seed collected. In the majority of cases, apparently normal quantities of seed were produced. However, several transformants (both antisense *Ms* and antisense *Icl* transformants) either produced no seed at all or very reduced quantities compared to controls. In such plants, fruit were reduced in size, the most severe reductions being in plants which produced no seed. The fruit of these plants characteristically failed to ripen and did not open. In addition, the entire flower (fruit and petals) frequently abscised together from the parent plant well before maturity, whilst in control and wild-type plants, the petals alone would abscise several days before the fruit would mature, desiccate and open (fruit in wild-type and control plants did not abscise). This phenotype was called the Low Seed Producing (LSP) phenotype. In its most extreme form, the LSP phenotype was clearly visible by both the morphology of the fruit, the early abscission from the plant leading to stems almost devoid of fruit and flowers, and the much reduced quantities of seed harvestable.



The LSP phenotype was more marked in plants of the  $T_1$  generation and data for the total quantities of seed produced by each of the 18  $T_1$  progeny of a  $T_0$  plant exhibiting the LSP phenotype (TICL 12.1) and that produced by the 20  $T_1$  progeny of a  $T_0$  plant producing apparently normal quantities of seed (TICL 7.1) were compared by applying Student's T-test. This resulted in a value of  $P$ , the probability that the difference of the mean weights of seed produced by the progeny of these plants was attributable to chance, of less than 0.001 (the probability that the data sets were significantly different is greater than 99.9 %). The LSP phenotype was therefore characterised at least in some plants by a statistically significant reduction in seed production compared to other transformants. Although the most extreme LSP plants (chiefly in the  $T_1$  generation) failed to produce any seed, most LSP plants, especially those in the  $T_0$  generation, bore a reduced number of fruit which exhibited the LSP phenotype to varying degrees, often producing seed but in reduced quantities.

#### **4.4.2 *The LSP phenotype appears to be caused by pollen inviability***

Among the *N. plumbaginifolia* *Ms* transformants of the  $T_0$  generation, two plants, TMS B.37.1 and TMS B.33.1, produced a mixture of fruit which abscised well before maturity, fruit which apparently reached maturity but failed to open or produce any seed, and fruit which were visibly smaller than wild-type or control plants but produced seed, albeit in reduced quantities (for technical reasons, data for quantities of seed produced by individual fruit was not obtainable).

The LSP phenotype was consistent with a failure in the process of sexual fertilisation. Because of the known involvement of the glyoxylate cycle in pollen formation (section 1.4.6), it was hypothesised that the LSP phenotype was a result of the antisense gene interfering with pollen formation, resulting in either defective pollen or a reduced quantity of viable pollen, which would lead to a failure in seed production. In order to test this, cross-pollination experiments were performed with flowers of the LSP  $T_0$  plants TMS B.37.1 and TMS B.33.1. Ten flowers each of TMS B.37.1 and of TMS B.33.1 were emasculated prior to anther dehiscence and the



stigma cross-pollinated with pollen from a wild-type plant. Conversely, ten flowers from a wild-type plant were emasculated, five cross-pollinated with pollen from TMS B.37.1 and five with that from TMS B.33.1. As a control, five flowers of TMS B.37.1 and five of a wild-type plant were emasculated but not cross-pollinated. All control flowers abscised before fruit had formed. The wild-type flowers cross-pollinated with pollen from the two LSP plants also abscised before formation of fruit. The TMS B.37.1 and B.33.1 flowers pollinated with wild-type pollen produced fruit of apparently normal size containing apparently normal quantities of seed. Thus, the LSP phenotype was largely rescued by wild-type pollen and wild-type flowers pollinated with pollen from LSP plants failed to develop fruit. This suggested strongly that the LSP phenotype was caused by pollen inviability.

#### **4.4.3 Seed from all *N. plumbaginifolia* Ms and *Icl* transformants was viable**

It was hypothesised that interference of the glyoxylate cycle with antisense genes would adversely affect embryogenesis or the ability of seed to mobilise lipid reserves and germinate. It was therefore anticipated that in a proportion of those antisense *N. plumbaginifolia* transformants producing seed, the seed might be inviable. Seed from all original T<sub>0</sub> transformants was tested for its ability to germinate and, simultaneously, for the presence in germinated seedlings of the *nptII* gene which confers kanamycin resistance. The *nptII* gene is part of pBI121. Cells showing kanamycin resistance were therefore transgenic and, assuming no rearrangements in the transferred DNA segment during or following transformation of plant tissue, contained the *Ms* or *Icl* cDNA fragments in the antisense orientation with respect to the CaMV 35S promoter. Based on the hypothesis that the LSP phenotype was caused by the presence and effective action of the antisense *Ms* or *Icl* genes in those plants, inviability was specifically anticipated for seed from LSP plants.

The results of germination tests on the T<sub>1</sub> seed produced by the original *Ms* and *Icl* T<sub>0</sub> transformants are shown in table 4.3 and 4.4, respectively. As can be seen from these data, all seed batches produced were viable. Although the proportions of



**Table 4.3: Germination of TMS T<sub>1</sub> seed.**

**kan<sup>r</sup>** : kanamycin-resistant. **kan<sup>s</sup>** : kanamycin-sensitive. **LBA4404 A.1.1**: control plant generated from transformation with untransformed strain LBA4404. **pBI121 A.1.1**: control plant generated from transformation with LBA4404 carrying the plasmid pBI121. All sample sizes were larger than 100 seedlings. Ratios are given correct to 2 significant figures. **Phenotype** column refers specifically to the LSP phenotype and has only been indicated for plants in which this was most apparent.

Self-fertilised T <sub>0</sub> parent:	Ratio kan <sup>r</sup> : kan <sup>s</sup> :	Phenotype:
LBA4404 A.1.1	all kan <sup>s</sup>	
pBI121 A.1.1	3.8	
TMS A.3.1	3.1	
TMS A.5.1	1.9	
TMS A.6.1	all kan <sup>s</sup>	
TMS A.7.1	0.55	
TMS A.11.2	3.4	
TMS A.12.1	3.9	
TMS B.3.1	3.3	
TMS B.3.2	23	LSP
TMS B.4.1	2.5	
TMS B.6.1	3.4	LSP
TMS B.8.1	3.8	
TMS B.8.2	2.8	
TMS B.8.3	3.8	
TMS B.9.1	29	
TMS B.10.1	2.8	
TMS B.11.1	2.9	
TMS B.12.1	3.4	
TMS B.12.2	16	LSP
TMS B.13.1	2.2	
TMS B.14.1	2.5	
TMS B.16.1	2.1	
TMS B.17.1	2.6	
TMS B.17.2	3.3	
TMS B.18.1	8.2	
TMS B.18.2	3.4	LSP
TMS B.18.3	3.5	
TMS B.19.1	11	
TMS B.19.3	0.030	LSP



Table 4.3, continued.

Self-fertilised T <sub>0</sub> parent:	Ratio kan <sup>r</sup> : kan <sup>s</sup> :	Phenotype:
TMS B.23.1	4.8	
TMS B.23.2	1.4	
TMS B.23.3	15	
TMS B.23.4	19	
TMS B.24.1	2.3	
TMS B.25.1	2.6	
TMS B.28.1	2.7	
TMS B.28.2	2.6	
TMS B.29.1	2.9	
TMS B.29.2	3.1	
TMS B.29.3	0.24	
TMS B.30.1	3.2	
TMS B.30.2	2.7	
TMS B.31.1	2.9	
TMS B.31.2	2.8	
TMS B.31.3	2.3	
TMS B.31.4	2.8	
TMS B.32.1	2.3	
TMS B.33.1	4.0	LSP
TMS B.33.2	4.1	
TMS B.34.1	3.6	
TMS B.34.2	2.2	
TMS B.34.3	3.0	
TMS B.35.1	3.3	
TMS B.35.2	3.9	
TMS B.37.1	3.2	LSP
TMS B.38.1	8.4	
TMS B.38.2	13	
TMS B.39.1	100	
TMS B.42.2	3.0	
TMS B.45.1	0.60	



**Table 4.4: Germination of TICL T<sub>1</sub> seed.**

Legend as for table 4.3. Control plants for the TICL set were generated separately from those in the TMS set.

Self-fertilised T <sub>0</sub> parent:	Ratio kan <sup>r</sup> : kan <sup>s</sup> :	Phenotype:
LBA4404.1.1	all kan <sup>s</sup>	
pBI121 1.1	2.9	
TICL 1.1	100	LSP
TICL 1.2	4.3	
TICL 1.3	2.1	
TICL 4.1	2.1	
TICL 7.1	12	
TICL 7.2	8.1	
TICL 9.1	2.7	
TICL 9.2	1.8	
TICL 11.1	2.6	
TICL 12.1	3.3	LSP
TICL 15.1	3.7	
TICL 18.2	2.2	
TICL 20.1	4.4	LSP
TICL 20.2	3.7	
TICL 20.4	2.6	
TICL 21.1	9.1	
TICL 21.2	23	
TICL 22.1	3.0	LSP
TICL 22.3	2.7	
TICL 24.1	22	
TICL 25.1	6.0	
TICL 25.2	10	LSP
TICL 28.1	2.2	LSP



seed germinated in all batches (including controls) appeared to be the same, there was considerable variability in the timing of germination both between and within batches, despite prior imbibition with GA<sub>3</sub> in an attempt to fully synchronise germination. All but one (TMS A.6.1) of the TMS and TICL batches contained kanamycin-resistant seedlings (kanamycin resistance was identifiable in seedlings at or after stage 7 of post-germinative growth). Kanamycin resistance is a dominant trait therefore insertion of a single copy of the transgene into the diploid *N. plumbaginifolia* genome should result in a T<sub>1</sub> seedling ratio of approximately 3 kanamycin-resistant : 1 kanamycin-sensitive. In many cases, the ratio of kanamycin-resistant to kanamycin-sensitive seedlings was close to 3:1, suggesting insertion of a single copy of the transgene. Where seed was from parent plants exhibiting the LSP phenotype, this has also indicated in tables 4.3 and 4.4. The two TMS LSP plants tested in cross-pollination experiments, TMS B.37.1 and TMS B.33.1, appear to carry a single transgene. If the LSP phenotype was due to inhibition of the glyoxylate cycle, this did not appear to affect germination in these plants.

Several batches of seed showed kanamycin resistance ratios which did not conform to the approximate 3:1 ratio. These fell into two categories. Batches with ratios significantly greater than 3 kanamycin-resistant : 1 kanamycin-sensitive include the TMS plants B.3.2, B.9.1, B.12.2, B.18.1, B.19.1, B.23.3, B.23.4, B.38.1, B.38.2 and B.39.1, and the TICL plants 1.1, 7.1, 7.2, 21.1, 21.2, 24.1, 25.1 and 25.2. These ratios may have been caused by the insertion into the genome of multiple unlinked copies of the transgene. The second category, in which the kanamycin-resistant : kanamycin-sensitive ratio was significantly smaller than 3:1, included the TMS plants A.7.1, B.19.3., B.23.2, B.23.3, B.29.3 and B.45.1. These ratios may have been caused by a bias against inheritance of the transgene.

The use of potato in the antisense *Ms* and *Icl* transformations was intended specifically to circumvent the anticipated problem of inviable seed being produced by the *N. plumbaginifolia* transformants, which would have made further analysis of transformant lines impossible. However, this did not occur. Because *N. plumbaginifolia* grows faster and is more amenable to genetic analysis than potato, it



was decided that no further analysis would be carried out on the potato transformants given the viability of transgenic *N. plumbaginifolia* seed.

#### **4.5 Assays of MS activity in T<sub>1</sub> seedlings of TMS transformants**

In order to identify TMS plants in which the antisense *Ms* gene was causing effective inhibition of MS synthesis, direct measurement of MS enzyme activity was carried out on all batches of T<sub>1</sub> seedlings derived from the self-fertilisation of original T<sub>0</sub> transformants. Seedlings at four days post-imbibition were harvested without stage-specific selection for MS assays. The enzyme activities obtained for duplicated seedling assays in this initial screen exhibited a large degree of variation which was attributed to asynchronous germination times within and between batches of seedlings. It was therefore decided that selection of seedlings specifically at stage 3 of post-germinative growth (see section 2.1.1) would be necessary to remove this element of variability. A selection of TMS T<sub>1</sub> seed batches were chosen for this second screen, seedlings allowed to germinate, those at stage 3 of post-germinative growth selected manually, and MS activity assays carried out on homogenates of these. Initial activities were corrected for the total protein concentrations of the homogenates. The results of this screen are shown in table 4.5. Assays for wild-type seedlings were carried out four times with separate aliquots of a single homogenate in order to establish the degree of variability inherent in the assay itself. In addition, two separate homogenates were prepared from seedlings of TMS A.11.2 and assayed separately to give an indication of the reproducibility of the homogenisation process, and two total protein assays were carried out on separate aliquots of a single homogenate from seedlings of TMS B.28.2 to give an indication of the variability inherent in the total protein assay (TMS A.11.2 and B.28.2 were chosen for this purpose at random). As can be seen in table 4.5, the majority of TMS sample readings fall outside the limits of the mean for wild-type  $\pm$  the standard error, both above and below. Although several of these showed activities lower than wild-type, several samples showed activities greater than wild-type.



If these values were an accurate representation of MS activity, those which showed increases in MS activity would have been of no interest with respect to the ultimate aims of the project, whilst no apparent reductions observed resulted in activities lower than 68 % of wild-type levels. It was decided that activity assays would not be used for further screening for the following reasons:

i) The four values obtained for repeated wild-type assays, carried out on separate aliquots of the same homogenate, show considerable variability. This indicated that the assay system itself was not reproducible to the necessary standard. In addition, correction of assay values for the total protein concentration of each homogenate was not sufficient to remove the element of irreproducibility introduced by the homogenisation process, as indicated by the high discrepancy between duplicate values for TMS A.11.2. The data shown in table 4.5 for transgenic seedling batches were obtained from single samples and assays (with the exceptions indicated). In order to make these data more reliable in light of the variability inherent in the assay system, several protein extractions, and several assays for each homogenate, would have been required for each batch of seedlings.

ii) Whilst antisense inhibition could result in a reduction of translatable mRNA, such a change would not necessarily be detectable by enzyme activity assays even if these were accurate.

It was therefore decided that TMS and TICL plants would continue to be screened by an alternative method. As no antibodies to MS or ICL from these species were available for immunological detection of protein levels (western blotting) in the TMS and TICL plants, and effects at the mRNA level may not result in effects detectable at the protein level, the method selected was RNA (northern) blotting. This was thought to be more amenable to standardisation between samples than enzyme activity assays and would reveal changes at the mRNA level.

Why?



**Table 4.5: MS activity assays in T<sub>1</sub> seedlings from T<sub>0</sub> parent plants**

<b>Self-fertilised parent of T<sub>1</sub> seedling batch assayed:</b>	<b>MS enzyme activity (<math>\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}</math>):</b>
wild-type (first assay)	16.30
wild-type (second assay)	11.45
wild-type (third assay)	10.45
wild-type (fourth assay)	10.55
TMS A.3.1	14.45
TMS A.11.2 (first assay)	17.67
TMS A.11.2 (second assay)	13.57
TMS B.6.1	10.67
TMS B.17.1	16.70
TMS B.17.2	8.29
TMS B.18.2	10.32
TMS B.18.3	9.98
TMS B.23.2	13.93
TMS B.23.3	9.66
TMS B.25.1	18.09
TMS B.28.2 (first assay)	18.66
TMS B.28.2 (second assay)	22.66
TMS B.29.1	10.14
TMS B.30.2	8.89
TMS B.31.2	17.27
TMS B.31.3	9.22
TMS B.35.1	10.05
TMS B.37.1	11.12
TMS B.38.2	9.15
TMS B.39.1	10.84
TMS B.42.1	10.72

Mean of four wild-type readings = 12.19  
Standard error (SE) = 1.39  
Mean - SE = 10.80  
Mean + SE = 13.58



## 4.6 Northern analysis of TMS and TICL tissue

### 4.6.1 Northern analysis of TMS $T_1$ seedlings

#### 4.6.1.1 *Ms* mRNA accumulates during post-germinative growth of wild-type *N. plumbaginifolia*

The developmental regulation of the *Ms* gene during post-germinative growth of *N. plumbaginifolia* was analysed by selection of wild-type seedlings from stages 0 to 7 (inclusive), extraction of total RNA, and northern hybridisation with a probe synthesised using the *Ms* cDNA fragment excised from pBlScTMS6 as template (hereafter referred to as the *Ms* probe). The result of this post-germinative time-course is shown in figure 4.3. As can be seen, a single transcript was visible, which corresponded to a size of approximately 2.5 kb when compared with migration of RNA size markers, detectable on the original agarose gel (not shown), and showed a pattern of expression during post-germinative growth which was very similar to that of the *Ms* transcript in other species (see section 1.4.3). Taken together, these data suggest strongly that this RNA species was the *Ms* transcript. The *Ms* mRNA was detectable in imbibed seed (stage 0), less so at stages 1 and 2, most abundant at stages 3 and 4, faintly detectable at stage 5 and undetectable at stages 6 and 7. This confirmed that *Ms* mRNA was most abundant at stage 3 in *N. plumbaginifolia*.

RNA levels  
during ger.

#### 4.6.1.2 Initial screening of TMS $T_1$ seedling batches by northern blotting

Seedlings at stage 3 of post-germinative growth were chosen as the most appropriate tissue for determining the steady-state levels of *Ms* mRNA in transgenic plants. Two criteria were available for determining which  $T_0$  TMS plants were most likely to have reduced levels of MS, and therefore whose  $T_1$  seedlings should be the first analysed by RNA blotting. These were:

- i) plants exhibiting the LSP phenotype (putative antisense phenotype).
- ii) plants whose seedlings gave the lowest values in MS activity assays.





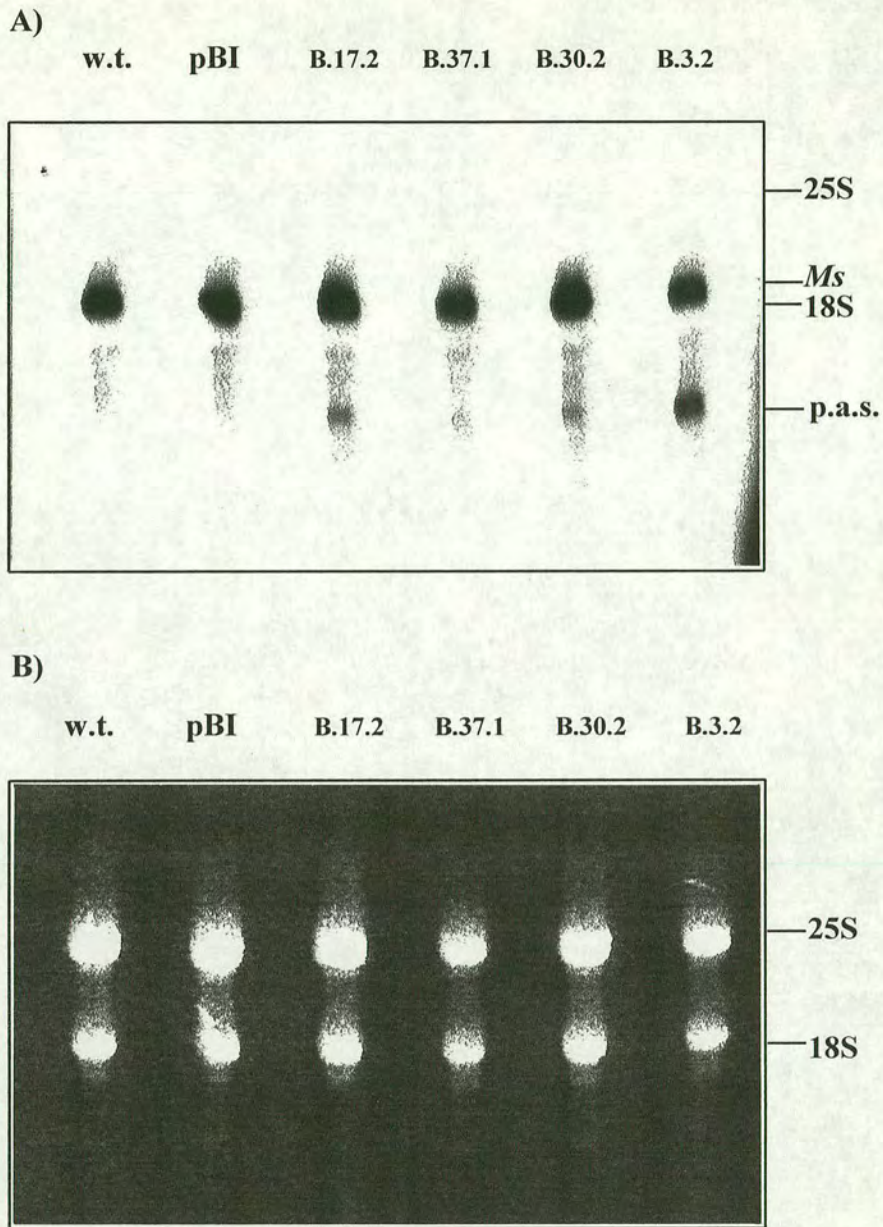
**Figure 4.3: Accumulation of *Ms* mRNA during post-germinative growth in *N. plumbaginifolia*.** Numbers correspond to the stages of post-germinative growth depicted in figure 2.1. Wild-type seedlings were allowed to grow in the light until they had reached the desired stage, were harvested and total RNA extracted. 2.5  $\mu$ g of RNA from seedlings at each stage was loaded in successive lanes and subjected to northern hybridisation with the *N. plumbaginifolia Ms* probe. **25S** and **18S**: positions of ribosomal RNA markers revealed by staining of the gel.



Neither of these criteria were known for certain to reflect the degree of antisense inhibition and therefore seedlings from two likely candidate plants in both categories were analysed. These were TMS B.37.1 and TMS B.3.2, which exhibited the LSP phenotype, and TMS B.17.2 and TMS B.30.2, which showed the lowest MS activity. It was known from kanamycin resistance tests that these plants carried the transgene (table 4.3). Stage 3 T<sub>1</sub> seedlings originating from these T<sub>0</sub> plants were selected and steady-state levels of *Ms* mRNA analysed by northern hybridisation with the *Ms* probe. The result of this experiment is shown in figures 4.4a and 4.4b. Figure 4.4a shows the result of the hybridisation and 4.4b, the corresponding agarose gel stained with ethidium bromide prior to blotting. In order to make valid comparisons of signals obtained from the hybridisation, equal loadings of total RNA in each lane were necessary. The ethidium bromide staining of the 25 s and 18 s (visible) ribosomal bands was assumed to be an accurate representation of total RNA, and this was subsequently confirmed (refer to section 4.6.1.4). As can be seen in figure 4.4b, the loadings in the gel depicted are indistinguishable.

In figure 4.4a, two sizes of band are visible. When compared with migration of RNA size markers and ribosomal RNA visible on the original agarose gel, the upper band (larger and most abundant transcript) corresponds to the *Ms* transcript (seen in figure 4.3) and the lower band (smaller and less abundant transcript) corresponds to approximately 800 nucleotides. This smaller band was only detectable in samples from TMS seedlings and not in the control samples. Taken together, these data suggested (but did not show directly) that this smaller RNA species was the *Ms* antisense transcript. Although the putative antisense band varied in intensity, the intensity of the *Ms* transcript in the TMS samples was similar to that in the control samples. This showed that accumulation of the *Ms* transcript had not been inhibited by the presence of the transgene (which conferred kanamycin resistance and may have provided the template for the smaller transcript) in the TMS seedlings tested. This result cast doubt both on the possibility that the LSP phenotype was caused by inhibition of the *Ms* gene (although this result with post-germinative seedlings does not exclude the possibility of antisense effects being significant in other stages





**Figure 4.4: TMS T<sub>1</sub> seedling screen, set 1.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5µg was loaded in each lane.

**A)** Northern blot hybridised with the *Ms* probe. **B)** Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. **Numbers above lanes** correspond to the parent TMS plant. **w.t.:** wild-type seedling RNA control. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. ***Ms*:** *Ms* mRNA. **p.a.s.:** putative antisense RNA. **25S** and **18S:** positions of ribosomal RNA markers.

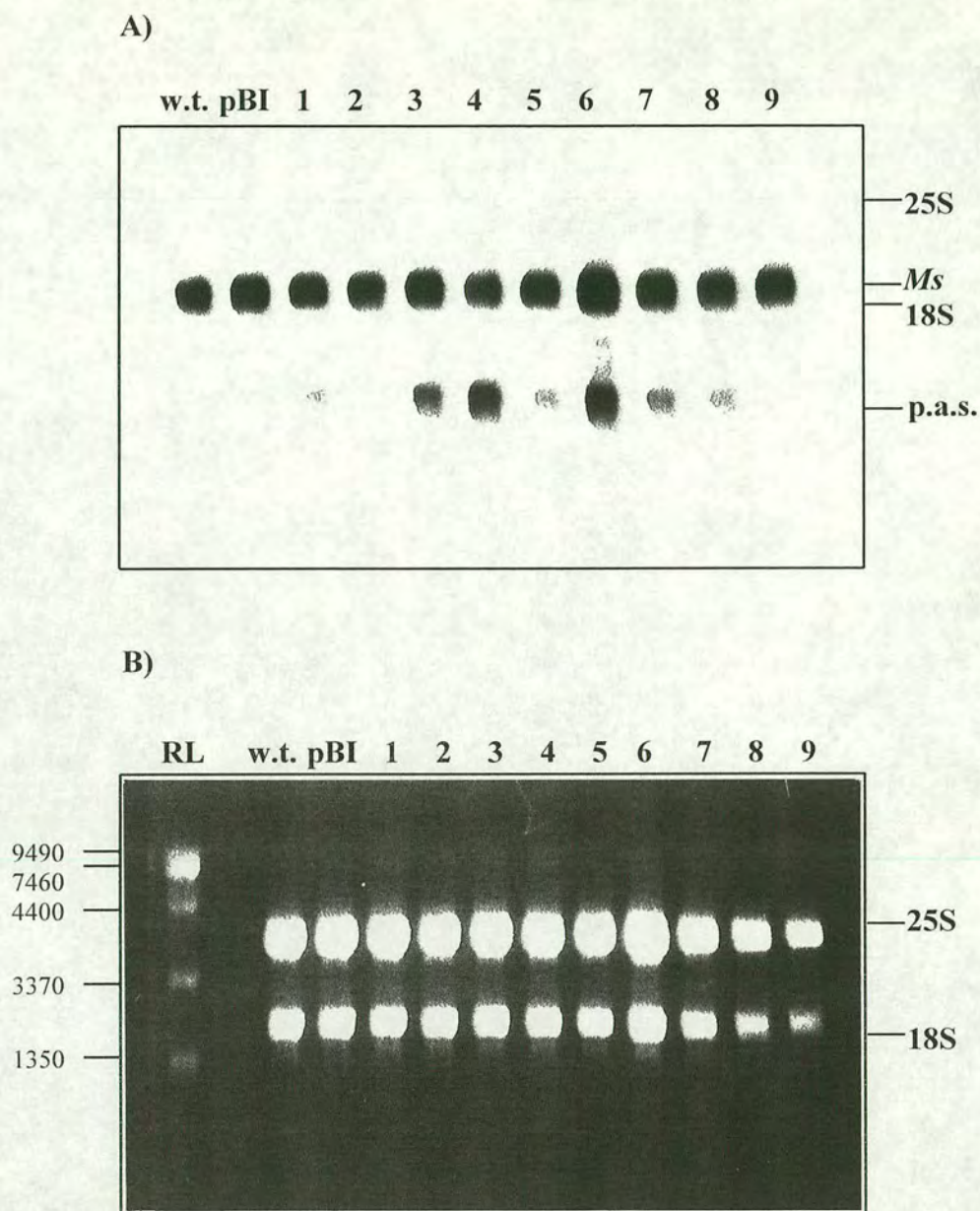


of development or tissues, such as pollen), and on the results obtained from the MS activity screen. It was therefore decided that all batches of  $T_1$  TMS seedlings would be screened by northern analysis without reference to either the LSP phenotype or results from the MS activity assays.

#### **4.6.1.3** *General screening of TMS $T_1$ seedling batches by northern blotting*

All batches of TMS  $T_1$  seedlings other than those screened in the northern blot depicted in figure 4.4 were screened by the same method. The results of these hybridisation experiments are shown in figures 4.5a, 4.6a and 4.7a, where the ribosomal band fluorescence on the corresponding agarose gels (shown in figures 4.5b, 4.6b and 4.7b, respectively) has been used as an indication of equal loading of total RNA within the set of samples electrophoresed on each individual gel. As can be seen, although most of the TMS  $T_1$  seedling batches showed signals no lower than either the controls or each other, slightly lower signals were observed in two notable cases, TMS B.23.3 (figure 4.5) and TMS B.19.3 (figure 4.7). The transfer of RNA, or its hybridisation, for TMS B.23.3 in figure 4.5 was clearly not even and in order to ascertain whether the altered signal in this lane was genuinely lower than other samples or simply a result of uneven hybridisation, blotting and hybridisation of TMS B.23.3 was repeated (refer to figure 4.7). Although to a degree the low signal for this sample in figure 4.5 was apparently due to anomalous hybridisation in this blot, figure 4.7 shows a signal for this sample very slightly lower than other samples, a difference which is more significant in light of the greater amount of total RNA loaded in this lane compared to other TMS sample lanes (also compare with the pBI121 control sample in this figure, where total RNA overloading is reflected by an obvious increase in the hybridisation signal).  $T_1$  seedlings descended from the  $T_0$  parent transformants TMS B.23.3 and TMS B.19.3 were therefore grown to maturity for identification and subsequent analysis of individual homozygous plants within the  $T_1$  generation.

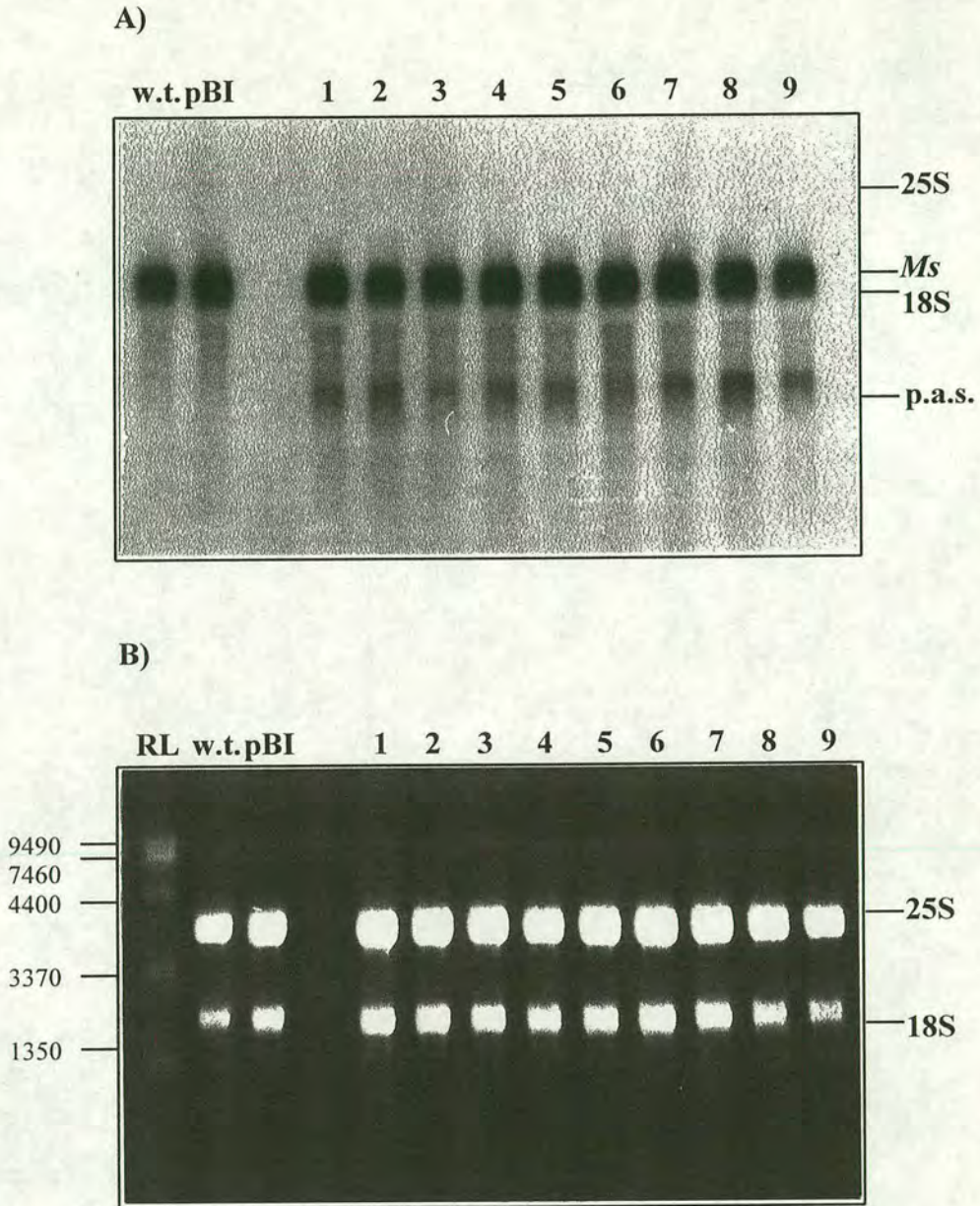




**Figure 4.5: TMS T<sub>1</sub> seedling screen, set 2.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5µg was loaded in each lane.

**A)** Northern blot hybridised with the *Ms* probe. **B)** Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. **Lane numbers** correspond to samples from the following TMS parent plants: **1:** A.11.2. **2:** B.18.2. **3:** B.18.3 **4:** B.23.3. **5:** B.31.3. **6:** B.38.2. **7:** B.35.1. **8:** B.42.1. **9:** A.6.1. **w.t.:** wild-type seedling RNA control. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. ***Ms*:** *Ms* mRNA. **p.a.s.:** putative antisense RNA. **25S** and **18S:** positions of ribosomal RNA markers. **RL:** RNA size markers, sizes in nucleotides are indicated.



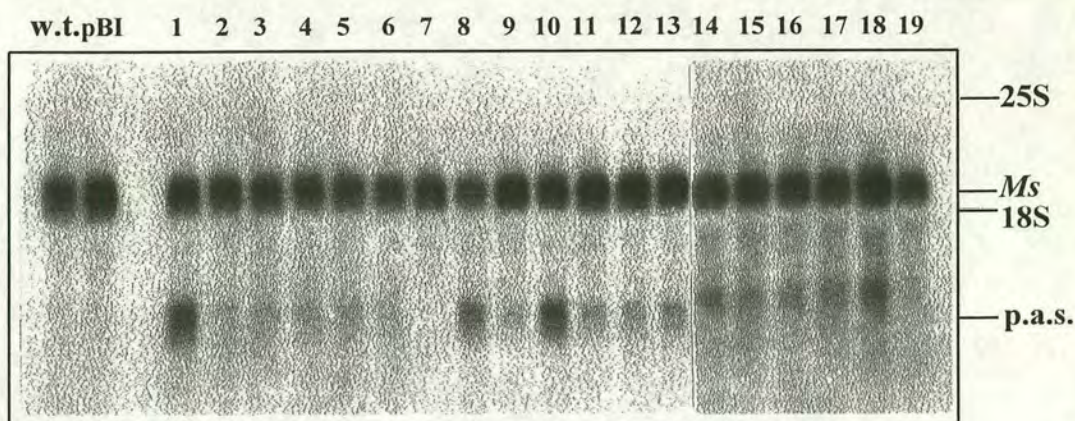


**Figure 4.6: TMS  $T_1$  seedling screen, set 3.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5 $\mu$ g was loaded in each lane.

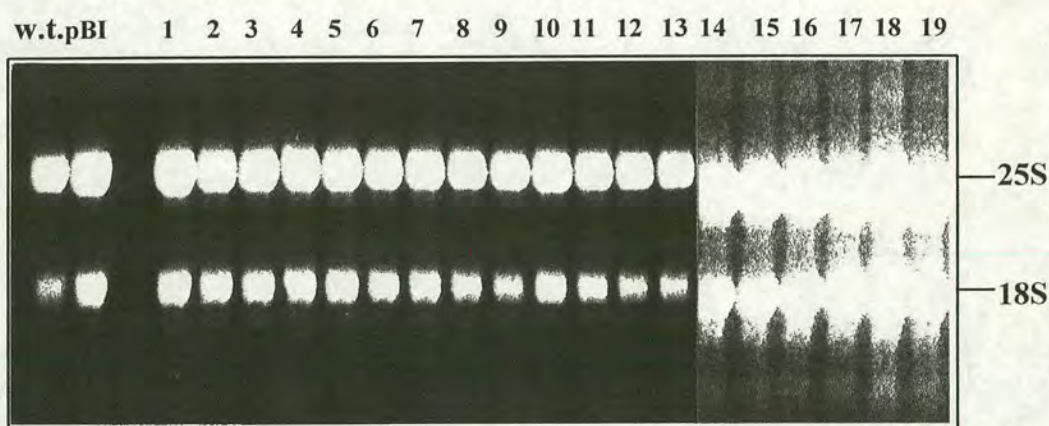
**A)** Northern blot hybridised with the *Ms* probe. **B)** Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. **Lane numbers** correspond to samples from the following TMS parent plants: **1:** B.28.2. **2:** B.12.2. **3:** A.7.1. **4:** B.6.1. **5:** B.8.1. **6:** B.33.1. **7:** B.29.1. **8:** B.39.1. **9:** B.34.1. **w.t.:** wild-type seedling RNA control. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. ***Ms*:** *Ms* mRNA. **p.a.s.:** putative antisense RNA. **25S** and **18S:** positions of ribosomal RNA markers. **RL:** RNA size markers, sizes in nucleotides are indicated.



A)



B)



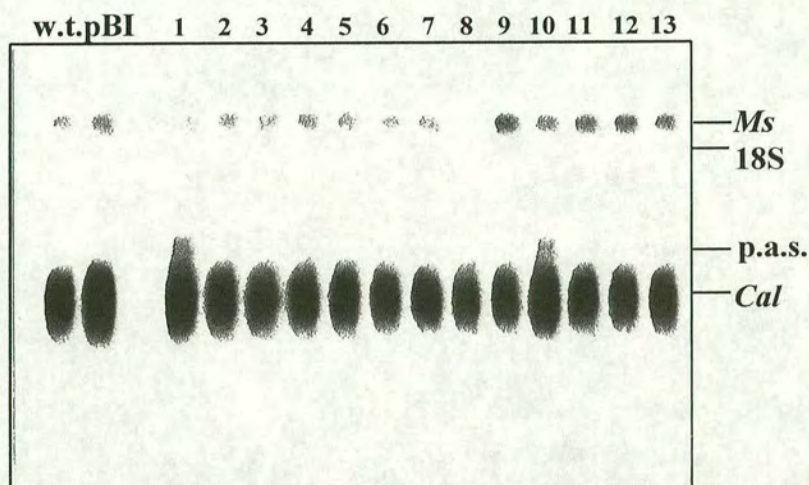
**Figure 4.7: TMS  $T_1$  seedling screen, set 4.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5 $\mu$ g was loaded in each lane. **A)** Northern blot hybridised with the *Ms* probe. **B)** Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. Note that the samples in lanes 14 to 19 were electrophoresed, blotted and hybridised separately from samples in preceding lanes and although 2.5 $\mu$ g of total RNA was loaded in all lanes, the photograph of the stained gel for samples 14 to 19 is more exposed than for the preceding samples. **Lane numbers** correspond to samples from the following TMS parent plants: **1:** B.23.3. **2:** B.20.1. **3:** A.3.1. **4:** B.25.1. **5:** B.41.1. **6:** B.43.1. **7:** A.5.1. **8:** B.19.3. **9:** B.13.1. **10:** B.9.1. **11:** B.16.1. **12:** B.10.1. **13:** B.4.1. **14:** A.12.1. **15:** B.11.1. **16:** B.14.1. **17:** B.24.1. **18:** B.32.1. **19:** B.45.1. **w.t.:** wild-type seedling RNA control. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. ***Ms*:** *Ms* mRNA. **p.a.s.:** putative antisense RNA. **25S** and **18S:** positions of ribosomal RNA markers.



#### 4.6.1.4 *Calmodulin mRNA steady-state levels reflect the levels of ribosomal RNA detectable by ethidium bromide fluorescence on RNA agarose gels*

In order to make valid comparisons of steady-state levels of the *Ms* transcript in different samples, it was necessary for loadings of total RNA on agarose gels prepared for northern blotting to be as near to equal as possible. Loadings of total RNA for the northern blots depicted in figures 4.3 up to and including 4.7 were determined prior to electrophoresis by spectrophotometry and confirmed following electrophoresis by the relative fluorescence of ethidium bromide bound to ribosomal RNA in each lane. In order to further confirm that the relative fluorescence of ribosomal RNA was a valid criterion for determining the relative amounts of total RNA, the steady-state levels of a transcript other than *Ms* were analysed. The mRNA to be selected for comparison had to be unrelated to *Ms* in terms of the biochemical role of the enzyme encoded, in order to ensure that any putative effects on the *Ms* gene, which might also influence expression of other genes in the glyoxylate cycle or gluconeogenesis, would not influence the comparison with ribosomal RNA. It also had to be of a distinguishable size to the *Ms* transcript in order to be easily identifiable on northern blots. For this purpose, a probe synthesised from a 534 bp potato calmodulin cDNA template (I. Oliver, University of Edinburgh) was used in a northern hybridisation with a blot previously hybridised with the *Ms* probe (the blot shown in figure 4.7). This blot was specifically chosen because it included samples of RNA isolated from seedlings derived from TMS B.23.3 and TMS B.19.3, the two batches of TMS seedlings which had been provisionally chosen for further analysis. The result of the hybridisation with the calmodulin probe is shown in figure 4.8. As can be seen, a single band was visible in addition to the *Ms* transcript and the putative antisense transcript. Its migration corresponds to that expected for the calmodulin transcript and it was concluded that this additional band was the calmodulin transcript. The relative intensities of the calmodulin bands correspond closely to the relative visible quantities of ribosomal RNA on the original agarose gel (figure 4.7b). This suggested strongly that visible levels of ribosomal RNA were a valid criterion for determining the equality of total RNA loading on northern blots and consequently provide a valid reference for comparison with steady-state levels of





**Figure 4.8: TMS  $T_1$  seedling screen, set 4, re-hybridised with a potato calmodulin probe.** The same blot as shown in figure 4.7, including controls and TMS sample lanes 1 to 13 only. **Lane numbers** correspond to samples from the following TMS parent plants (as for lanes 1 to 13 in figure 4.7): **1:** B.23.3. **2:** B.20.1. **3:** A.3.1. **4:** B.25.1. **5:** B.41.1. **6:** B.43.1. **7:** A.5.1. **8:** B.19.3. **9:** B.13.1. **10:** B.9.1. **11:** B.16.1. **12:** B.10.1. **13:** B.4.1. **w.t.:** wild-type seedling RNA control. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. **Cal:** calmodulin mRNA. **Ms:** *Ms* mRNA. **p.a.s.:** putative antisense RNA. **18S:** position of ribosomal RNA marker.



the *Ms* transcript. In addition, this result clearly reconfirmed that the lane corresponding to TMS B.23.3 had been overloaded relative to the other TMS sample lanes and that the signal observed was therefore an over-representation. This suggested that the level of the *Ms* transcript in seedlings derived from TMS B.23.3 was genuinely low, as proposed in section 4.6.1.3. Because it was anticipated that putative antisense effects detected in T<sub>1</sub> seedlings would be more marked in the T<sub>2</sub> seedlings of homozygous T<sub>1</sub> transformants, and would allow confirmation of the results obtained with T<sub>1</sub> seedlings, these results were not quantified at this stage.

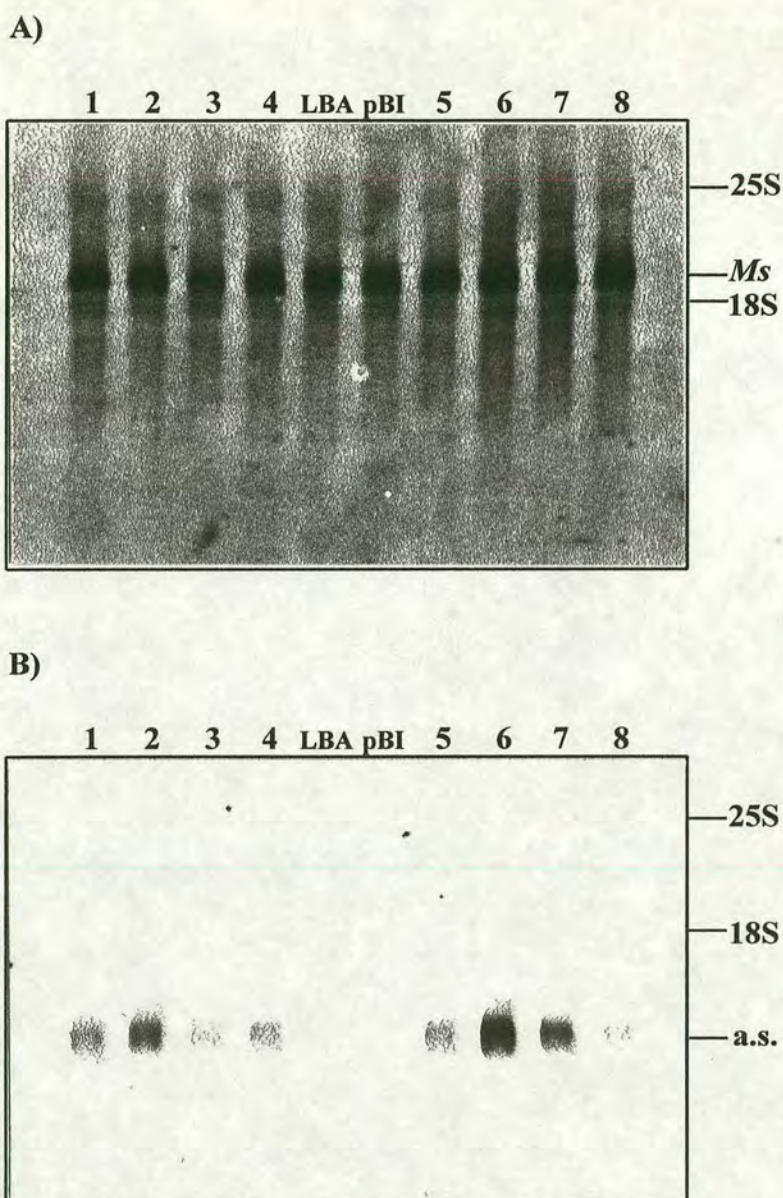
#### **4.6.1.5** *The smaller RNA species detectable in TMS samples in hybridisations with the Ms probe was antisense RNA*

In the majority of TMS T<sub>1</sub> seedling RNA samples, a second band other than the *Ms* mRNA was visible following probing with the *Ms* probe. As described in section 4.6.1.2, this band was only ever present in RNA samples prepared from TMS seedlings (never controls) and was of a size consistent with the suggestion that the antisense cDNA fragment was its template. It was proposed in section 4.6.1.2 that this band may be the antisense RNA. In order to test this, single-stranded RNA probes (riboprobes) were synthesised by *in-vitro* transcription using pBlScTMS6 (see section 4.2.1) as template. Riboprobes incorporating the immunologically detectable epitope DIG were synthesised from either end of the *N. plumbaginifolia* *Ms* cDNA fragment in this plasmid, resulting in labelled RNA molecules complementary to either the sense or antisense strands of the corresponding segment of *Ms* coding sequence. In this experiment, therefore, the riboprobe synthesised by transcription in the sense orientation should detect specifically antisense sequences, whilst the riboprobe synthesised by transcription in the antisense orientation should detect specifically sense sequences. However, for convenience, the riboprobe **which detects sense RNA** will hereafter be referred to as the **Sense** probe and, conversely, the riboprobe **which detects antisense RNA** will hereafter be referred to as the **Antisense** probe.



The intensity of the putative antisense band varied widely between samples and was apparently absent in a few cases (for example, TMS A.5.1 in figure 4.7). Eight TMS  $T_1$  seedling RNA samples were chosen for hybridisation with the riboprobes by virtue of the strong intensity of the putative antisense signal in those samples when hybridised with the double-stranded *Ms* probe. The samples chosen were those extracted from seedlings originating from the  $T_0$  parent plants TMS B.3.2, B.9.1, B.12.2, B.18.3, B.19.3, B.23.3, B.38.2 and B.39.1 (note that in addition to this criterion, the seedling batches from TMS B.19.3 and B.23.3 were those selected for further generation of  $T_2$  seedlings, whilst TMS B.3.2, B.12.2 and B.19.3 were plants notable for displaying the LSP phenotype). Northern hybridisations were carried out with two identical blots of these samples. One blot was hybridised with the Sense riboprobe, the other with the Antisense riboprobe. The result of this experiment is shown in figure 4.9. Hybridisation with the Sense riboprobe (figure 4.9a) resulted in the appearance of a single band identically in all lanes, including controls, which was taken to be the *Ms* mRNA. Hybridisation with the Antisense riboprobe (figure 4.9b), however, resulted in the appearance of a smaller band only (re-hybridisation of each blot with the converse riboprobe was not possible due to technical reasons). The intensity of hybridisation varied in accordance with the intensities of the putative antisense bands in previous hybridisations with the double-stranded *Ms* probe, migrated at the same position relative to the *Ms* mRNA and was absent from control lanes. It was concluded that this band was the same as the putative antisense band detected in previous hybridisations with the double-stranded *Ms* probe. This band was antisense RNA complementary to the fragment of *N. plumbaginifolia* cDNA in pBIScTMS6 used both to synthesise the *Ms* probe and in the construction of the plant transformation vector pBITMS1 (see section 4.2.2). Taken together, these data suggest strongly that the antisense RNA originated from transcription of the antisense cDNA fragment from the CaMV 35S promoter. It was concluded that in seedlings in which the additional band was clearly detectable, the transgene was being correctly and efficiently expressed, leading to accumulation of antisense *Ms* RNA.





**Figure 4.9: Hybridisations of TMS T<sub>1</sub> seedling RNA with single-stranded *Ms* RNA probes.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5µg was loaded in each lane. **A)** Hybridisation with **Sense** probe (probe complementary to the sense strand). **B)** Hybridisation with **Antisense** probe (probe complementary to antisense strand). **Lane numbers** correspond to samples from the following TMS parent plants: **1:** B.3.2. **2:** B.9.1. **3:** B.12.2. **4:** B.18.3. **5:** B.19.3. **6:** B.23.3. **7:** B.38.2. **8:** B.39.1. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. ***Ms*:** *Ms* mRNA. **a.s.:** antisense *Ms* RNA. **25S** and **18S:** positions of ribosomal RNA markers.



Contrary to previous results with the double-stranded *Ms* probe, the *Ms* mRNA signal in the TMS B.19.3 and B.23.3 samples (figure 4.9a) did not appear lower than controls (nor did it in any other TMS sample tested with the Sense riboprobe). However, in the light of previous results and the fact that the quantities of total RNA on the blots probed with the riboprobes had not been specifically adjusted to be equal (unlike previous hybridisations), it was decided to continue analysis of TMS B.23.3 and B.19.3 despite this. In addition, the samples extracted from seedlings originating from parent plants clearly exhibiting the LSP phenotype (aside from B.19.3 which was also selected for further analysis on the strength of hybridisation results), TMS B.3.2 and B.12.2, did not give particularly strong antisense signals (figure 4.9b) compared to the other TMS samples tested and showed apparently normal *Ms* mRNA signals (figure 4.9a). This again put into doubt any putative causative link between the transgene and the LSP phenotype.

#### **4.6.2 Strategies for enhancing the effect of the antisense *Ms* transgene**

The lack of any apparent negative correlation between the relative strengths of antisense and sense (*Ms* mRNA) signals in figure 4.9 suggested that either the antisense *Ms* RNA was ineffective (in the quantities detected) in reducing steady-state levels of *Ms* mRNA in stage 3 T<sub>1</sub> seedlings compared to wild-type, as was proposed in section 4.6.1.2, or that it was effecting a reduction which was undetectable due to the mixture of wild-type, heterozygous and homozygous transgenic seedlings in the T<sub>1</sub> generation. Analysis of plants homozygous for the transgene may have revealed an otherwise undetectable reduction. If, however, the antisense *Ms* RNA (in the quantities detected) was genuinely ineffective in reducing steady-state levels of *Ms* mRNA in stage 3 T<sub>1</sub> seedlings, then this could have been due to either of two reasons:

i) The antisense *Ms* RNA was having no effect at all on the steady-state level of *Ms* mRNA. In this case, a stoichiometric excess of antisense RNA would have



been no more effective in reducing *Ms* mRNA steady-state levels than the stoichiometric deficit observed.

ii) The antisense RNA had an effect on *Ms* mRNA steady-state levels, but only when in stoichiometric excess to the *Ms* mRNA. This situation would have been likely if the mechanism of antisense inhibition is assumed to involve hybridisation of sense with antisense RNA. As can be seen, in hybridisation experiments with TMS T<sub>1</sub> seedling RNA (figures 4.4 to 4.9, inclusive), the antisense RNA, though in some cases strongly expressed, was never in excess with respect to the sense transcript. This may have been the reason for a lack of apparent reduction of mRNA levels compared to controls.

Assuming the latter of these two explanations to be applicable, the stoichiometric deficit of antisense RNA may have been due to the extremely high levels of expression of the *Ms* gene during post-germinative growth. Because the CaMV 35S promoter would be expected to be inactive in dry seed, whilst the *Ms* gene is highly expressed immediately following imbibition of seed (see figure 4.3), *Ms* mRNA may have begun to accumulate at an earlier stage than antisense RNA. This was subsequently confirmed (refer to section 4.6.3.4). The steady-state levels of *Ms* mRNA may therefore have always been in excess of antisense RNA during this stage of seedling development. This might have precluded any potential effect of the antisense RNA.

In order to investigate circumstances in which the expression of the transgene was not effectively delayed with respect to the *Ms* gene in this way, two strategies were devised:

i) Investigation of the effect of the antisense transgene in green seedlings following starvation treatment. The CaMV 35S promoter should be active in photosynthetically competent seedlings whilst the *Ms* promoter was inactive (refer to section 1.4.3). Subsequent starvation treatment should induce expression of the *Ms* gene (refer to sections 1.4.5 and 1.5.2.2). Under these circumstances, antisense RNA would already be present at a time when *Ms* transcripts may be only just beginning to



accumulate and may be more effective in inhibiting the accumulation of mRNA to the levels which would otherwise be reached in starvation conditions.

ii) Investigation of the effect of the antisense transgene in tissues in which *Ms* is expressed, other than seedlings immediately following germination. One appropriate tissue for such an investigation is anther tissue, as the *Ms* gene is known to be expressed in pollen (refer to section 1.4.6). If the antisense transgene were concomitantly active in pollen, it would not be subject to the delay of expression which may have been occurring during post-germinative growth.

These approaches are discussed in the following two sections.

#### **4.6.2.1 Starvation treatment of TMS seedlings**

In order to allow antisense RNA to accumulate to higher levels than *Ms* transcripts, TMS seedlings were grown until 9 days after imbibition and subsequently incubated in darkness for five days prior to harvesting and total RNA extraction. This stage of development was chosen because it fell well after *Ms* mRNA from the post-germinative growth response was no longer detectable (figure 4.3) but before *N. plumbaginifolia* cotyledons become naturally senescent, which was found to be approximately 25 days after imbibition. Induction of starvation in these seedlings should therefore have allowed accumulation of antisense RNA in the absence of *Ms* mRNA prior to induction of *Ms* gene expression by starvation.

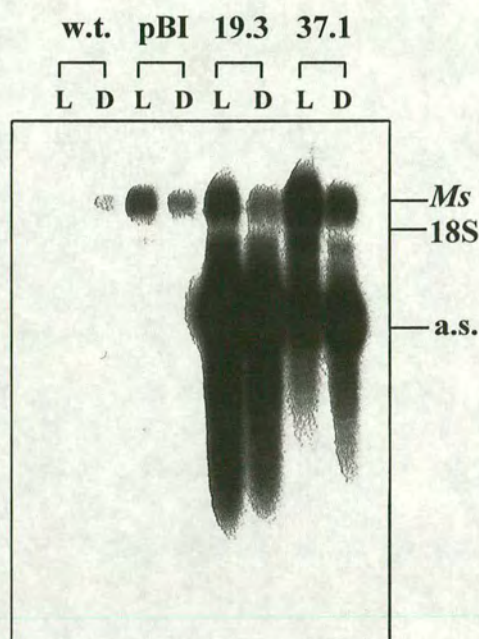
Two TMS samples were chosen for this experiment, a sample in which antisense RNA steady-state levels were known to be high and the *Ms* mRNA levels thought to be low according to initial general screening results (TMS B.19.3) and a sample derived from seedlings originating from a  $T_0$  plant exhibiting the LSP phenotype (TMS B.37.1. Although evidence up to this point had suggested that the LSP phenotype was unconnected to the transgene, this remained to be proven). In order to enrich the  $T_1$  seedling sample for transformed seedlings, thereby maximising the detectable effect of the transgene on *Ms* mRNA steady-state levels, the TMS seedlings were germinated on medium containing kanamycin and only seedlings



which remained apparently healthy (green) after 14 days were harvested. Two controls were carried out in this experiment, dark-incubation of wild-type and pBI121 seedlings. In order to make the pBI121 control as comparable as possible to the TMS samples, the same kanamycin selection procedure was applied to pBI121 seedlings (also T<sub>1</sub> generation). The result of a hybridisation of these samples with the *Ms* probe is shown in figure 4.10. The autoradiograph shown has been deliberately overexposed to show faint signals. As can be seen from the wild-type samples, dark incubation caused slight accumulation of *Ms* transcripts compared to light. However, all other samples showed a considerably more significant increase than this in the light (lanes 3, 5, 7) compared to the levels apparent in corresponding samples from dark-incubated seedlings (lanes 4, 6, 8). The only difference in the treatment given to wild-type seedlings as opposed to that given to the pBI121 control and TMS seedlings was the kanamycin selection step. From the data obtained in figure 4.10, it must be concluded that the presence of kanamycin causes an induction of *Ms* gene expression in the light.

The induction of *Ms* expression caused by darkness in this experiment was therefore surpassed by this unexpected synergistic effect. However, the original purpose of the experiment, to create circumstances under which the antisense RNA could accumulate to levels surpassing those of the *Ms* transcript, were nevertheless achieved. The antisense RNA was clearly more abundant than the *Ms* transcript in both light and dark-incubated TMS seedling samples (lanes 5 to 8). The levels of antisense RNA relative to *Ms* mRNA were considerably greater than those observed in hybridisations with samples from the same seedling batches at stage 3 of post-germinative growth (figures 4.4 and 4.7). This apparent increase could have been due to several reasons. *Ms* mRNA in this experiment may have been much less abundant than at stage 3 of post-germinative growth and therefore the increase in antisense RNA levels relative to previous hybridisations may only be an apparent increase. Alternatively, the increase was genuine and may have been due either to a greater abundance of antisense RNA at 14 days post-imbibition than at stage 3 of post-germinative growth, or because kanamycin induces expression of the transgene, in the light and dark, though this latter explanation would seem unlikely as kanamycin





**Figure 4.10: Starvation treatment of TMS T<sub>1</sub> seedlings.** Seedlings were allowed to germinate and were either grown in light until 14 days after imbibition and then harvested, or incubated in the light for 9 days followed by darkness for 5 days and harvested (14 days total). Total RNA was extracted and subjected to northern analysis with the *Ms* probe. 2.5µg of total RNA was loaded in each lane. **w.t.:** wild-type seedling RNA control. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. **19.3:** RNA from seedlings descended from TMS B.19.3. **37.1:** RNA from seedlings descended from TMS B.37.1. **L:** seedlings grown in light. **D:** dark-treated seedlings. **Ms:** *Ms* mRNA. **a.s.:** antisense *Ms* RNA. **18S:** position of ribosomal RNA marker.



would not be expected to influence expression of the transgene in addition to the *Ms* gene and yet do so in a qualitatively different manner. Which of the three explanations given above was applicable could not be established, as this hybridisation and previous hybridisations, with stage 3 seedling RNA, were not comparable. However, it was not necessary to resolve this question in order to determine the effect of the antisense RNA on *Ms* transcript levels in this experiment. Despite an overabundance of antisense RNA in the light-incubated TMS B.19.3 sample, the steady-state level of *Ms* mRNA was no lower, in fact appeared greater, than that in the pBI121 (light-incubated) control sample (total RNA loadings in all lanes were equal). It was therefore concluded that an excess of antisense RNA did not inhibit accumulation of *Ms* mRNA in this experimental system.

#### 4.6.2.2 Analysis of TMS $T_1$ anther tissue

The second approach to investigate the effect of the antisense transgene in circumstances other than seedlings undergoing post-germinative growth, was northern analysis of anther tissue from mature  $T_1$  TMS transformants. Reports (van der Meer *et al.*, 1989; Plegt & Bino, 1989) have suggested that the CaMV 35S promoter is not active in the tapetum or sporogenic cells of anthers. However, it was not known for certain that the transgene would not be expressed, whilst expression of the endogenous *Ms* gene was known to be at a low level in pollen (Zhang *et al.*, 1994), increasing the probability of successful stoichiometric competition. In addition, anther tissue was the only tissue available in which the *Ms* gene was known to be active other than post-germinative seedlings or tissue undergoing starvation. It was therefore assumed for the purposes of the experiment that the *Ms* gene and the antisense *Ms* transgene would be concomitantly active during pollen development and it was anticipated that this would allow the accumulation of antisense RNA relative to that of the *Ms* mRNA to be greater than in post-germinative seedlings.

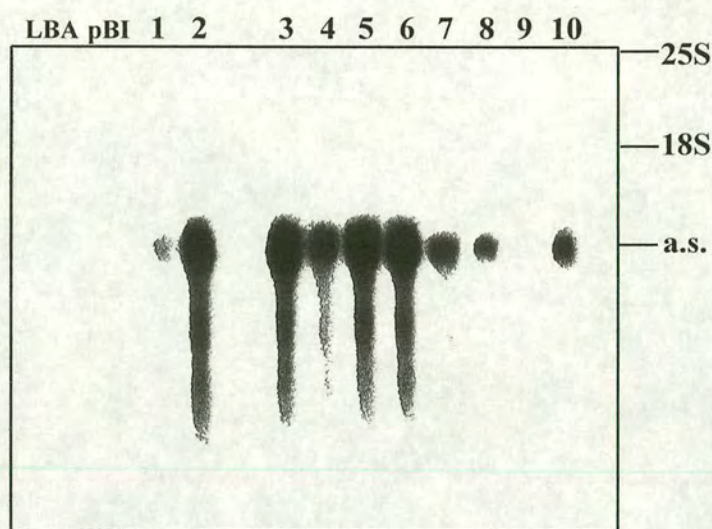
The plants selected for this analysis fell into several partially overlapping categories. The first category included  $T_1$  plants originating from seedling batches



which were thought to have shown lower steady-state levels of the *Ms* mRNA than controls. The T<sub>1</sub> plants TMS B.19.3.1, B.19.3.5, B.19.3.9 and B.23.3.7 fell into this class. TMS B.19.3.1 also represented the second category, apparently normal (non-LSP) plants descended from T<sub>0</sub> transformants which exhibited the LSP phenotype, whilst B.23.3.7 represented a third category, of plants which did not show the LSP phenotype and were descended from non-LSP T<sub>0</sub> plants. The final category included plants which were not only descendants of T<sub>0</sub> transformants which exhibited the LSP phenotype, but also exhibited the LSP phenotype themselves. This included TMS B.3.2.1, B.3.2.4, B.3.2.7, B.19.3.5, B.19.3.9, B.33.1.5 and B.37.1.19. It should be noted that the seed from the T<sub>1</sub> plants whose anther tissue was analysed had not been tested for kanamycin resistance at the time this experiment was carried out. Consequently the choice of plants was made without reference to whether they were known for certain to be transformants. As a result, one of the plants tested, TMS B.33.1.5, was subsequently discovered to be a non-transformant and this was reflected in the result obtained in the anther tissue northern analysis, as discussed below (the significance of this with respect to the LSP phenotype is discussed in section 4.6.3.2). Anthers were harvested from flowers at a stage prior to dehiscence which was identified by the appearance of the flowers, as described in section 2.1.1. However, the size of the flowers was not an accurate indication of the developmental stage of the anther contained within. Anthers close to or at the point of dehiscence were brown in colour whilst immature anthers were a range of shades of green, with the youngest most pale. The colour of anthers harvested from flowers of the same size often differed. However, due to the subjective nature of assessing the colour of anthers at different stages of maturity, the more quantifiable measurement of flower proportions had to be retained as the method for selecting developmental stage in spite of this. Equal loadings of anther total RNA were electrophoresed, transferred to nylon membrane and hybridised with the *Ms* probe as described above.

The result of this hybridisation is shown in figure 4.11. It can be seen in this figure that the *Ms* mRNA was not detectable, even in the LBA4404 and pBI121-





**Figure 4.11: Northern analysis of TMS T<sub>1</sub> anther tissue.** Undehisced anthers from flowers of the same size were harvested, total RNA extracted and subjected to northern analysis with the *Ms* probe. 20 µg of total RNA was loaded in each lane. **Lane numbers** correspond to anther samples from the following T<sub>1</sub> TMS plants: **1:** B.19.3.1. **2:** B.23.3.7. **3:** B.3.2.1. **4:** B.3.2.4. **5:** B.3.2.7. **6:** B.19.3.9. **7:** B.3.2.9. **8:** B.19.3.5. **9:** B.33.1.5. **10:** B.37.1.19. **LBA:** anthers from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **pBI:** anthers from a control plant carrying T-DNA from the pBI121 plasmid. **a.s.:** antisense *Ms* RNA. **25S** and **18S:** positions of ribosomal RNA markers.



transformed control samples. Only the antisense RNA was detectable and the intensity of this signal varied between the TMS samples. At the stage of anther development examined, therefore, no determination of the effect of antisense RNA could be made as the *Ms* mRNA itself was not detectable. Because of this, and the difficulty of selecting anthers of exactly the same developmental stage, no further analysis of TMS anther tissue was carried out. Note that the antisense RNA was not detectable in the sample from B.33.1.5. This would be expected in light of the fact that no seedlings descended from B.33.1.5 were subsequently found to be kanamycin-resistant (refer to section 4.6.3.2) and this plant therefore probably did not contain the transgene, or was not expressing it.

### **4.6.3 Analysis of TMS $T_2$ seedlings**

#### **4.6.3.1 Propagation of TMS transformants**

Of the original  $T_0$  TMS transformants, five were chosen for further analysis. These were TMS B.3.2, B.19.3, B.23.3, B.33.1 and B.37.1. Twenty  $T_1$  seedlings descended from each of these plants were selected at random and grown to maturity, with the exception of TMS B.23.3, from which fifteen were grown. The T-DNA was expected to segregate in these populations to give a 3:1 ratio of transgenic to non-transgenic seedlings in cases with a single T-DNA insertion site. However, wild-type seedlings could not be eliminated by kanamycin selection as even kanamycin resistant seedlings could not be recovered for further growth in compost. It was therefore expected that some seedlings chosen would be found not to carry the transgene. An advantage associated with this was that wild-type plants segregating from each population could subsequently serve as controls. Descendants were named by adding the number of the individual  $T_1$  plant (between 1 and 20, or 1 and 15 for the descendants of TMS B.23.3) to the name of the original  $T_0$  transformant. Thus,  $T_1$  descendants of TMS B.3.2 were named TMS B.3.2.1 to B.3.2.20, those of TMS B.19.3 were B.19.3.1 to B.19.3.20, those of TMS B.23.3 were B.23.3.1 to B.23.3.15,



those of TMS B.33.1 were B.33.1.1 to B.33.1.20 and those of TMS B.37.1 were B.37.1.1 to B.37.1.20.

The  $T_0$  transformants chosen for propagation were selected for several reasons. From hybridisations of  $T_1$  seedling RNA with the *Ms* probe, they were known all to express the antisense *Ms* RNA (refer to figures 4.4a, 4.6a and 4.7a). In the case of TMS B.23.3 and B.3.2, the antisense signal was particularly strong and the steady-state level of *Ms* mRNA in samples from B.19.3 and B.23.3 was apparently lower than controls. In addition, TMS B.3.2, B.19.3, B.33.1 and B.37.1 exhibited the LSP phenotype.

The  $T_1$  descendants of these plants were grown to maturity and seed collected. The  $T_2$  seed from all plants (samples of 100 seeds or more) was germinated on medium containing kanamycin to score seedlings for resistance. The batches of seed from TMS B.3.2.1 to .20, B.33.1.1 to .20 and B.37.7.1 to .20 exhibited a range of ratios of kanamycin-resistant seedlings : kanamycin-sensitive seedlings, including several ratios larger than 15:1, which suggested incomplete segregation of multiple unlinked copies of the transgene.

Of the 15 batches of seed from TMS B.23.3.1 to .15, 5 batches were completely kanamycin-resistant, 7 batches gave resistant : sensitive ratios of approximately 3:1, 1 batch gave a ratio of approximately 15:1 and 1 batch (B.23.3.11) gave a ratio of 0.025:1. The anomalous 0.025:1 ratio may have been caused by conditions specific to B.23.3.11 which led to an effect similar to that observed in plants descended from B.19.3, as discussed below. If the B.23.3.11 batch data is disregarded, the remaining data suggested the possibility that the  $T_0$  parent B.23.3 may have carried two unlinked copies of the transgene, although do not fit closely to the Mendelian ratio expected if this had been the case. However, this possibility seems likely given the original kanamycin resistance data of the mixed  $T_1$  seed produced by B.23.3, which gave a resistant : sensitive ratio of 15:1 (refer to table 4.3).



Most seed batches from TMS B.19.3.1 to .20 gave resistant : sensitive ratios close to 3:1 or smaller, as low as 0.3:1 (B.19.3.5). TMS B.19.3.3 produced only kanamycin resistant seed, whilst TMS B.19.3.4, B.19.3.7 and B.19.3.16 produced only kanamycin sensitive seed. The high (10 out of 20) frequency of the 3:1 ratio, alongside ratios below 3:1, suggested that the  $T_0$  parent TMS B.19.3 may have carried a single copy of the transgene but that there may have been a bias against inheritance or expression of the transgene in these seedlings. Two pieces of evidence support the latter explanation:

i) The  $T_1$  seed produced by TMS B.19.3 itself gave a very low resistant : sensitive ratio of 0.03:1 (refer to table 4.3). If this had been a reflection of the inheritance of the transgene, the frequency of propagated  $T_1$  plants subsequently producing kanamycin-resistant  $T_2$  seed would have been expected to be very low. In fact, 17 out of the 20  $T_1$  plants propagated produced at least some kanamycin-resistant seed.

ii) Scoring of kanamycin-resistant  $T_2$  seedlings from TMS B.19.3.1 to .20, unlike other seedling batches, was an ambiguous process. Seedlings which initially appeared to be resistant frequently underwent chlorosis typical of sensitive seedlings after a period of 7 to 14 days after germination, in a manner that suggested initial expression of the kanamycin resistance gene and subsequent loss of expression.

If there had been a bias against expression of the transgene in  $T_1$  and  $T_2$  seedlings generated from TMS B.19.3, this must have been an effect specific to germination in the presence of kanamycin (or to the expression of the kanamycin resistance marker gene as opposed to the CaMV 35S promoter-antisense *Ms* fusion) as hybridisation experiments with  $T_1$  seedling RNA demonstrated synthesis of antisense RNA (refer to figure 4.7).



#### **4.6.3.2** *The LSP phenotype in T<sub>1</sub> plants does not always co-segregate with the kanamycin resistance marker gene*

The appearance of the mature T<sub>1</sub> plants descended from TMS B.3.2, B.19.3, B.23.3, B.33.1 and B.37.1, with respect to the LSP phenotype, was noted. A particularly large proportion (17 plants out of 20) of the T<sub>1</sub> descendants of TMS B.19.3 exhibited the LSP phenotype to some degree, although all batches of T<sub>1</sub> plants included some plants which exhibited the LSP phenotype, except TMS B.23.3.1 to .20, which did not include any.

When comparing the data for kanamycin resistance of T<sub>2</sub> seedling batches with the LSP phenotype in their T<sub>1</sub> parents, it was apparent from the data for some plants that the LSP phenotype was not dependent on inheritance of the *nptII* gene (and hence in all probability the antisense transgene in the absence of recombination between the two). In five cases (TMS B.37.1.17, B.37.1.18, B.37.1.13, B.37.1.8 and B.33.1.5), plants clearly exhibiting some or all symptoms characteristic of the LSP phenotype produced exclusively kanamycin-sensitive seed (three plants from the B.19.3.1 to .20 set also may have been in this category, but the T<sub>2</sub> kanamycin resistance scoring process was not clear enough in this set to make this assertion). These plants cannot have been carrying the kanamycin resistance gene (or their T<sub>2</sub> descendants were not expressing it). This suggested strongly that the LSP phenotype was not caused by the presence of the transgene. Therefore, further analysis of TMS plants was carried out without reference to the LSP phenotype.

#### **4.6.3.3** *Northern analysis of TMS T<sub>2</sub> seedlings*

In order to select TMS T<sub>2</sub> seedling batches for northern analysis, reference was made to the apparent steady-state levels of *Ms* mRNA detected in northern hybridisations with T<sub>1</sub> TMS seedlings (refer to figures 4.4 to 4.7). As discussed in section 4.6.1.3, the two T<sub>1</sub> seedling batches in which inhibition of *Ms* mRNA accumulation appeared most likely were those from TMS B.19.3 and B.23.3. In order to maximise the



detectability of potential differences in mRNA steady-state levels in samples prepared from batches of T<sub>2</sub> seedlings, only batches which contained exclusively transgenic (kanamycin resistant) seedlings were selected. These were TMS B.23.3.1, .2, .4, .10, .13 and B.19.3.3. Total RNA was extracted from stage 3 T<sub>2</sub> seedlings descended from these plants for northern hybridisation. Aside from the standard LBA4404 control used in all previous hybridisations with stage 3 seedling RNA (total RNA extracted from stage 3 seedlings descended from a plant regenerated from leaf discs infected with untransformed LBA4404), two new controls were employed:

i) Seedling RNA from a plant (PCOR 1A; S. M. Smith, personal communication) known to be homozygous with respect to a transgene carried within the pBIPCOR plasmid (Graham *et al.*, 1990). This plasmid contains the *gus* gene under the control of a cucumber *Ms* promoter fragment. Seedling RNA from this plant was originally thought to be a control appropriate to the TMS T<sub>2</sub> seedling RNA analysis as PCOR 1A seedlings were homozygous for a transgene whereas only mixed T<sub>1</sub> seed was available for the pBI121-transformed control. It was therefore thought PCOR 1A seedlings would be most similar to TMS seedlings homozygous for the antisense *Ms* transgene. However, the PCOR 1A control was not ideal, as the presence of the *Ms* promoter fragment could have potentially led to co-suppression effects, making results for this control incomparable with those from TMS samples. In addition, transformation and growth conditions of PCOR 1A plants were not identical to those for the TMS plants and other controls.

ii) The second new control was RNA from stage 3 T<sub>2</sub> seedlings descended from TMS B.23.3.11. This T<sub>2</sub> batch contained mainly kanamycin sensitive seedlings (resistant : sensitive ratio 0.025:1), thus B.23.3.11 was considered to be the plant likely to have the closest genetic similarity to a non-transformed (effectively wild-type) descendant of TMS B.23.3 available. However, though at a low frequency, the progeny produced by B.23.3.11 did contain kanamycin resistant seedlings. None of the descendants of TMS B.23.3 grown to maturity produced exclusively kanamycin sensitive seed. The validity of this control is discussed further, below.

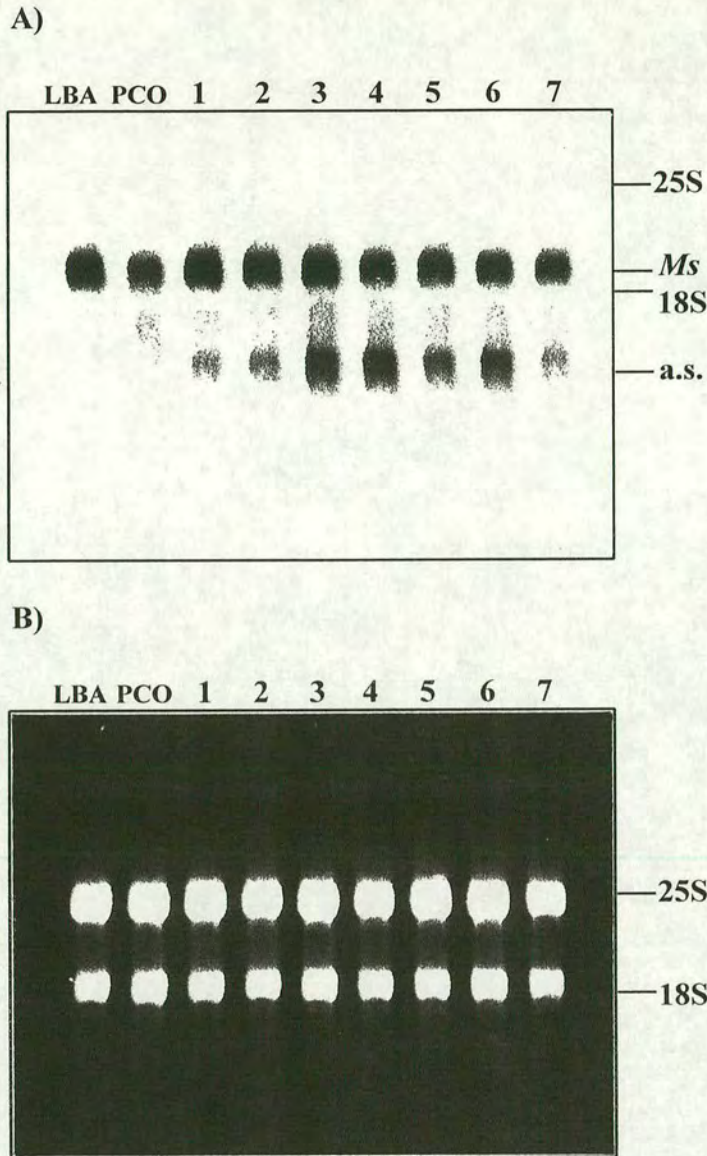


Equal loadings of total RNA from stage 3 seedlings of the six TMS members of interest and the three control plants were electrophoresed (see figure 4.12b), the RNA transferred to nylon membrane and hybridised with the *Ms* probe. The result of this hybridisation is shown in figure 4.12a. As can be seen, the *Ms* mRNA signal was not significantly lower in any of the samples compared to the LBA4404 control. The *Ms* mRNA steady-state level in the TMS B.19.3.3 sample was very slightly lower, reflected by an equally small inequality in the quantity of total RNA loaded (figure 4.12b), and in the PCOR 1A sample (figure 4.12a) despite equal loading of total RNA. As a control, the PCOR 1A sample would not have been expected to have *Ms* mRNA levels any lower than the LBA4404 control, but its validity as a control is questionable (discussed above).

As with RNA extracted from  $T_1$  seedlings, which were a mixture of homozygous, heterozygous and wild-type, the additional antisense band was clearly present in the TMS  $T_2$  samples (figure 4.12a). That this band in  $T_2$  seedlings was the same antisense *Ms* RNA as in the  $T_1$  seedlings was reconfirmed by the use of riboprobes. Figure 4.13a shows a blot of the same TMS samples as figure 4.12 (with the exception of B.19.3.3 for which no more RNA was available) probed with the Sense probe and 4.13b, with the Antisense probe. The hybridisation with the Sense probe allowed only the *Ms* mRNA band to be detected (in all samples) whilst the Antisense probe only hybridised to the small additional band exclusively present in the TMS samples. This reconfirmed that the smaller band was antisense RNA, as was shown for  $T_1$  seedlings, and showed heritability and stable expression of the transgene over successive generations.

The antisense *Ms* RNA was detectable in all TMS samples (refer to figure 4.12a) and particularly so in the TMS B.23.3.2, .4, .10 and .13 samples. It should be noted that the antisense band was detectable, albeit faintly, in the sample extracted from seedlings descended from TMS B.23.3.11, despite the fact that when tested for kanamycin resistance, the number of kanamycin sensitive seedlings from this plant was 40-fold greater than resistant seedlings. This suggested that in seedlings of

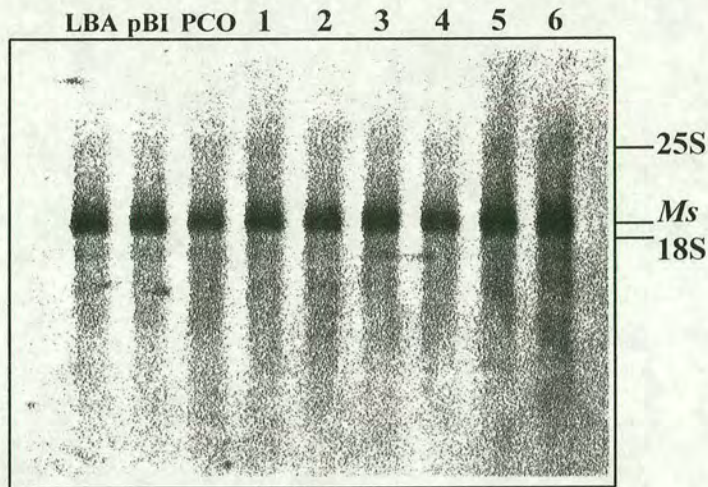




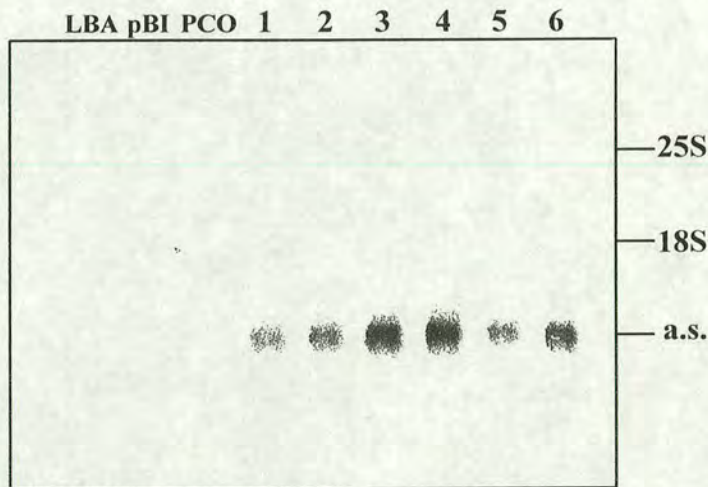
**Figure 4.12: Northern analysis of TMS  $T_2$  seedlings.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5 $\mu$ g was loaded in each lane. **A)** Northern blot hybridised with the *Ms* probe. **B)** Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. **Lane numbers** correspond to the following  $T_1$  TMS parent plants: **1:** B.23.3.11. **2:** B.23.3.1. **3:** B.23.3.2. **4:** B.23.3.4. **5:** B.23.3.10. **6:** B.23.3.13. **7:** B.19.3.3. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **PCO:** control sample from seedlings carrying T-DNA from the pBIPCOR plasmid. ***Ms*:** *Ms* mRNA. **a.s.:** antisense *Ms* RNA. **25S** and **18S:** positions of ribosomal RNA markers.



A)



B)



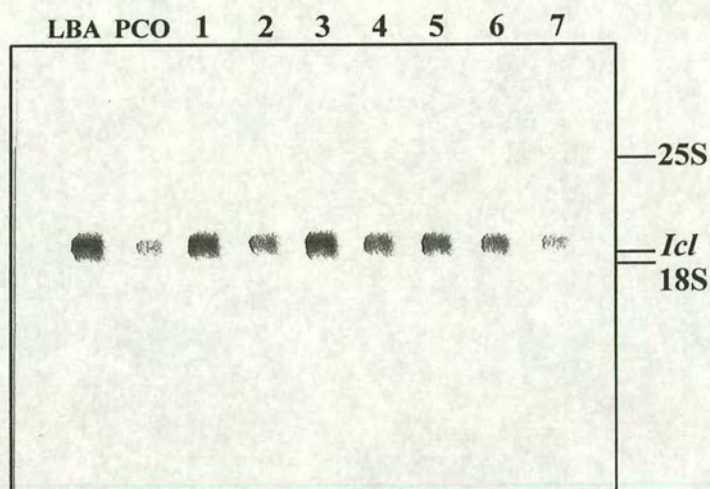
**Figure 4.13: Hybridisations of TMS  $T_2$  seedling RNA with single-stranded *Ms* RNA probes.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5 $\mu$ g was loaded in each lane. **A)** Hybridisation with **Sense** probe (probe complementary to the sense strand). **B)** Hybridisation with **Antisense** probe (probe complementary to antisense strand). **Lane numbers** correspond to samples from the following  $T_1$  TMS parent plants: **1:** B.23.3.11. **2:** B.23.3.1. **3:** B.23.3.2. **4:** B.23.3.4. **5:** B.23.3.10. **6:** B.23.3.13. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. **PCO:** control sample from seedlings carrying T-DNA from the pBIPCOR plasmid. ***Ms*:** *Ms* mRNA. **a.s.:** antisense *Ms* RNA. **25S** and **18S:** positions of ribosomal RNA markers.



B.23.3.11, either the presence of kanamycin inhibited expression of the complete transgene (both the *nptII* gene and the CaMV 35S promoter-antisense cDNA fusion), that the *nptII* gene was not active in most of these seedlings whilst the CaMV 35S promoter was, or that the few seedlings which did exhibit kanamycin resistance synthesised so much antisense RNA that it compensated for the majority of seedlings, which themselves synthesised none.

The slightly lower *Ms* mRNA steady-state level observed in the PCOR 1A sample compared to the LBA4404 control could have been specific to *Ms* mRNA only, or an effect common more generally to the expression of other glyoxylate cycle genes also. Establishing which of these was the case would give an indication of the influence of differences in growth conditions, or the degree of variability inherent in the seedling selection process, with respect to the steady-state levels of *Ms* mRNA levels detectable by northern hybridisation. This was considered significant because it would give an indication of whether the small differences in *Ms* mRNA steady-state levels observed were a reliable measure of the potential effects of the transgene. Because all the observed differences were small, a small degree of variability in the experimental system (with reference to differences in the seedling selection process and growth conditions in particular) may have had a relatively large influence on the observed changes. In order to establish whether the apparently low *Ms* mRNA level in the PCOR 1A sample was specific to *Ms*, or more general, the same blot which was depicted in figure 4.12a was stripped of the *Ms* probe and re-hybridised with the *Icl* probe (synthesised using the *N. plumbaginifolia* *Icl* cDNA fragment excised from pBIScTICL10 as template, refer to section 4.2.1). The result of this re-hybridisation is shown in figure 4.14. The signal observed was specific to *Icl* mRNA because following removal of the *Ms* probe, but prior to re-hybridisation, no signal was detected (result not shown). In addition, the antisense *Ms* RNA was clearly not detected by hybridisation with the new probe. As can be seen, the slight difference in *Ms* mRNA signal in the PCOR 1A sample compared to the LBA4404 control visible in figure 4.12a was also observed following hybridisation with the *Icl* probe. This suggested that a degree of variability was inherent in the experimental system. Although this was apparently minor, it suggested that the detection of very





**Figure 4.14: TMS T<sub>2</sub> seedling RNA, re-hybridised with the *N. plumbaginifolia* *Icl* probe.** The same blot as shown in figure 4.12, following removal of the *Ms* probe and re-hybridisation with the *Icl* probe. **Lane numbers** correspond to the following T<sub>1</sub> TMS parent plants (as in figure 4.12): **1:** B.23.3.11. **2:** B.23.3.1. **3:** B.23.3.2. **4:** B.23.3.4. **5:** B.23.3.10. **6:** B.23.3.13. **7:** B.19.3.3. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **PCO:** control sample from seedlings carrying T-DNA from the pBIPCOR plasmid. *Icl*: *Icl* mRNA. **25S** and **18S:** positions of ribosomal RNA markers.

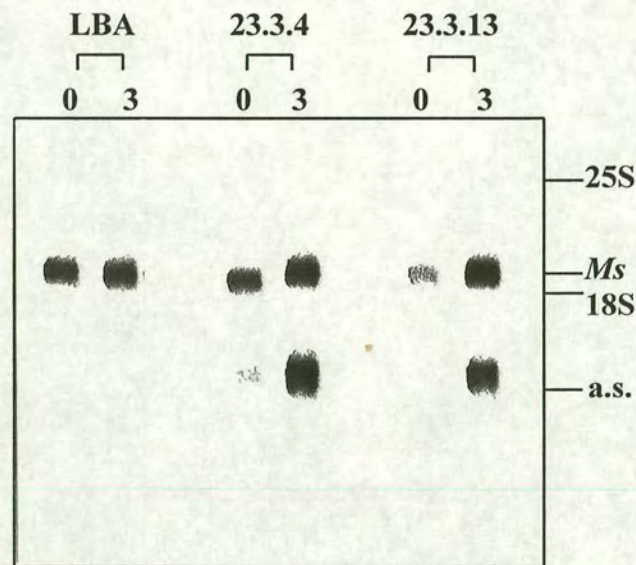


small changes in *Ms* mRNA steady-state levels was not a reliable indication of effects specific to the antisense transgene. The *Icl* mRNA signals in the TMS samples were also an exact reflection of the *Ms* mRNA signals observed in figure 4.12a. This confirmed that *Ms* mRNA steady-state levels were not significantly lower in any of the transformants studied compared to the LBA4404 control, despite detectable accumulation of antisense *Ms* RNA.

#### **4.6.3.4** *Ms mRNA in TMS seedlings accumulates more rapidly during post-germinative growth than does the antisense Ms RNA*

The failure of the antisense *Ms* RNA to cause any detectable inhibition of *Ms* mRNA accumulation in stage 3 TMS seedlings may have been due to *Ms* mRNA accumulating more rapidly than antisense RNA in seedlings following imbibition. Accumulation of *Ms* mRNA to high levels prior to the accumulation of significant levels of antisense RNA may have precluded the antisense RNA having any effect. In order to establish the relative rates of accumulation of *Ms* mRNA and antisense RNA, a northern blot of total RNA extracted from stage 0 and stage 3 seedlings was hybridised with the *Ms* probe. Total RNA from stage 0 and stage 3 TMS B.23.3.4 and B.23.3.13 were compared (the antisense *Ms* RNA was known to be clearly detectable in stage 3 total RNA samples from these plants). The result of this hybridisation is shown in figure 4.15. The amount of *Ms* mRNA relative to antisense RNA was clearly greater at stage 0 than at stage 3 for both TMS B.23.3.4 and B.23.3.13. This suggested strongly that *Ms* mRNA accumulated more rapidly than the antisense *Ms* RNA during post-germinative growth. If relative stoichiometric amounts of sense and antisense RNA are important in determining the effect of the antisense RNA, this delay may have been the reason why the antisense *Ms* RNA appeared to be ineffective in causing any detectable inhibition of *Ms* mRNA accumulation in the TMS plants.





**Figure 4.15: Comparison of *Ms* mRNA and antisense RNA steady-state levels at stage 0 and stage 3 of post-germinative growth.** Stage 0 seeds and stage 3 seedlings were harvested, total RNA extracted and subjected to northern analysis with the *Ms* probe. 2.5 µg of total RNA was loaded in each lane. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **23.3.4:** RNA from seed and seedlings descended from TMS T<sub>1</sub> parent plant B.23.3.4. **23.3.13:** RNA from seed and seedlings descended from TMS T<sub>1</sub> parent plant B.23.3.13. **0:** RNA sample from seed at stage 0 of post-germinative growth. **3:** RNA sample from seedlings at stage 3 of post-germinative growth (refer to figure 2.1). ***Ms*:** *Ms* mRNA. **a.s.:** antisense *Ms* RNA. **25S** and **18S:** positions of ribosomal RNA markers.



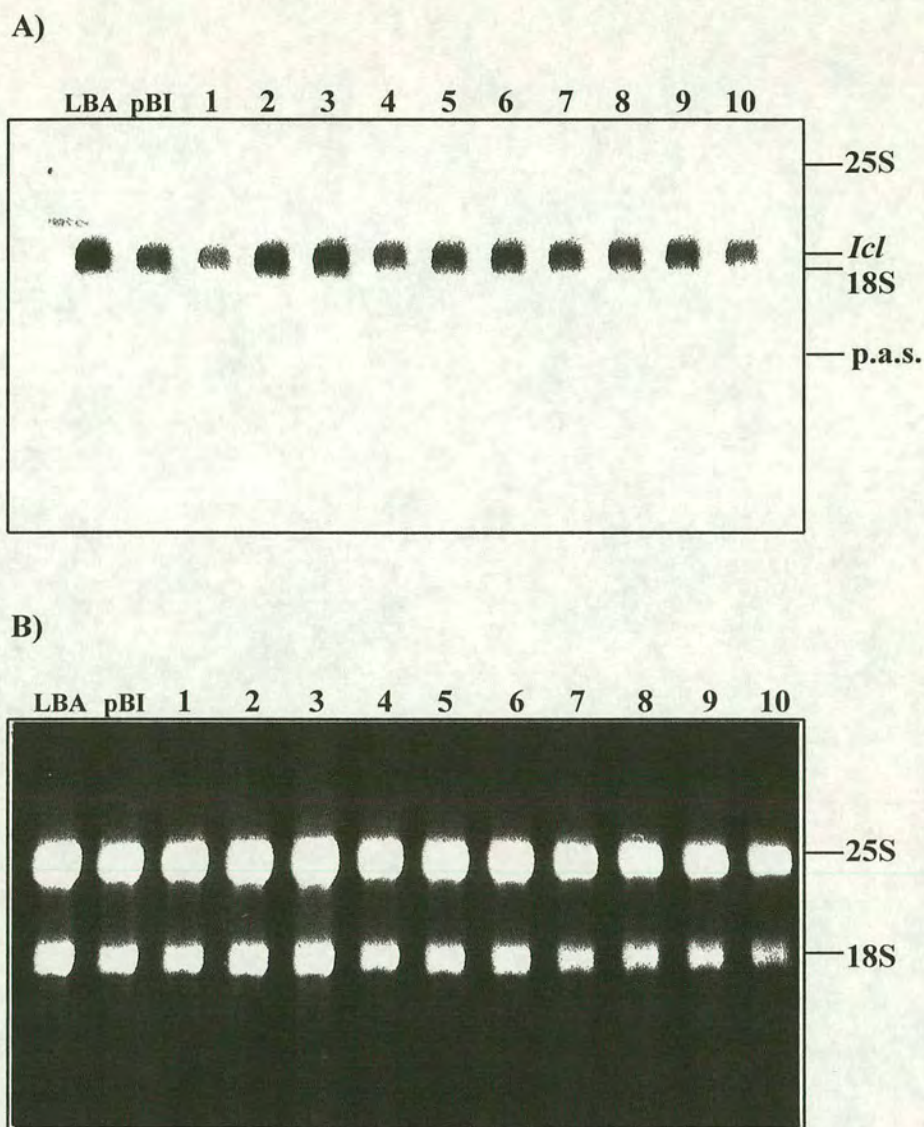
#### 4.6.4 Northern analysis of TICL seedlings

##### 4.6.4.1 Analysis of TICL $T_1$ seedlings

The  $T_1$  seedlings descended from all TICL  $T_0$  transformants (refer to table 4.4; all  $T_0$  TICL transformants produced at least some kanamycin resistant seed) were analysed in northern blot hybridisations with the *Icl* probe by the same method as the TMS seedling batches (refer to section 4.6.1.2). All twenty-three TICL batches were screened in three northern hybridisations. Adjustment of total RNA loadings for the first two hybridisations was carried-out solely on the basis of spectrophotometric estimates of total RNA concentration and were only approximately equal. The third hybridisation was carried-out twice in order to adjust loadings to be more precisely equal, based on the relative fluorescence of ribosomal RNA bands. Samples in which the steady-state levels of *Icl* mRNA appeared low in the first two hybridisations were re-analysed in the third hybridisation. The results of these three hybridisations are shown in figures 4.16a, 4.17a and 4.18a, with the agarose gel corresponding to the blot depicted in figure 4.18a shown in figure 4.18b (the result for the sample in the final lane, TICL 25.2, was disregarded, as the amount of total RNA was significantly less than in other lanes. This was due to TICL 25.2 having produced a very small quantity of seed and consequently little RNA being available). Three samples from the first two blots, TICL 1.1 and 7.1 (figure 4.16) and 21.1 (figure 4.17) appeared to show lower *Icl* mRNA levels than controls and were re-analysed in the third hybridisation following adjustment of total RNA loadings. As can be seen from figure 4.18a, TICL 1.1 and 21.1 still appeared to have levels of *Icl* mRNA lower than controls, whilst 7.1 did not. In addition, TICL 20.1 also appeared to have a lower *Icl* mRNA signal. A small additional band was visible in the TICL sample lanes. Although this band was not demonstrated to be the antisense RNA in the way the antisense *Ms* RNA band in TMS samples was, the size and frequency of appearance of the additional band in TICL samples suggested that it was, similarly, antisense *Icl* RNA originating from the transgene.

In order to establish whether these differences were specific to the *Icl* mRNA, the blot depicted in figure 4.18a was stripped of the *Icl* probe and re-

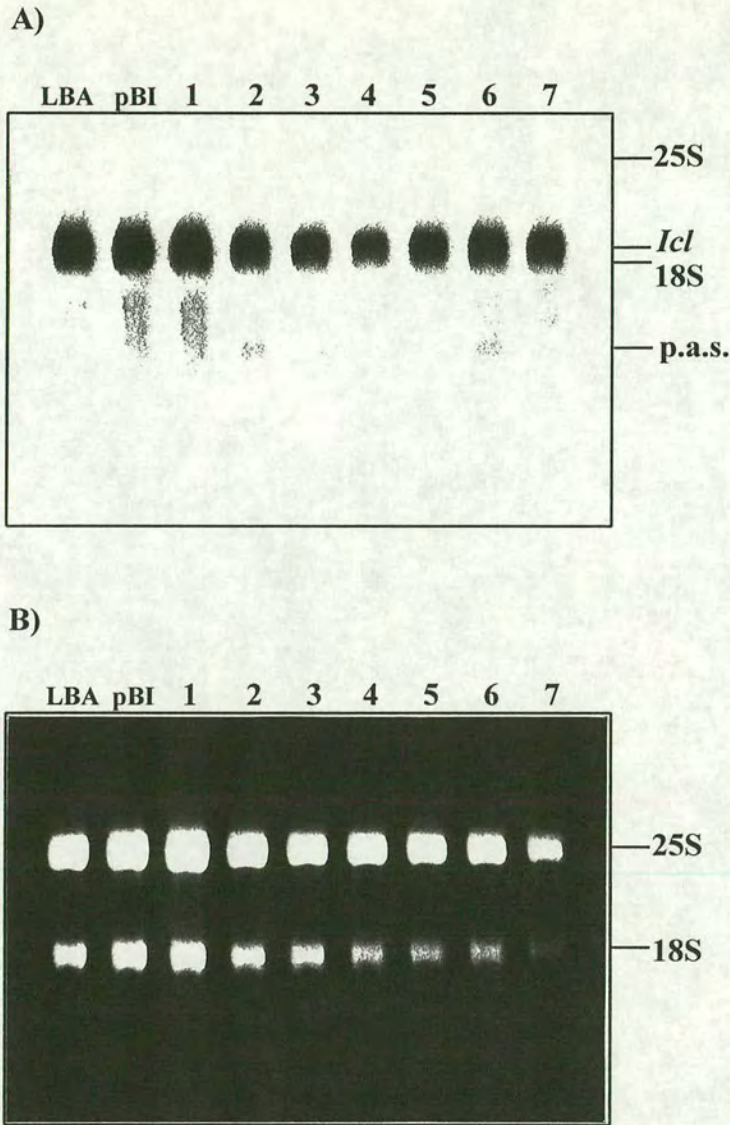




**Figure 4.16: TICL T<sub>1</sub> seedling screen, set 1.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5µg was loaded in each lane.

A) Northern blot hybridised with the *Icl* probe. B) Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. **Lane numbers** correspond to samples from the following TICL parent plants: **1:** 1.1. **2:** 1.2. **3:** 4.1. **4:** 7.1. **5:** 9.1. **6:** 11.1. **7:** 15.1. **8:** 18.2. **9:** 20.4. **10:** 22.1. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. *Icl*: *Icl* mRNA. **p.a.s.:** putative antisense *Icl* RNA. **25S** and **18S:** positions of ribosomal RNA markers.

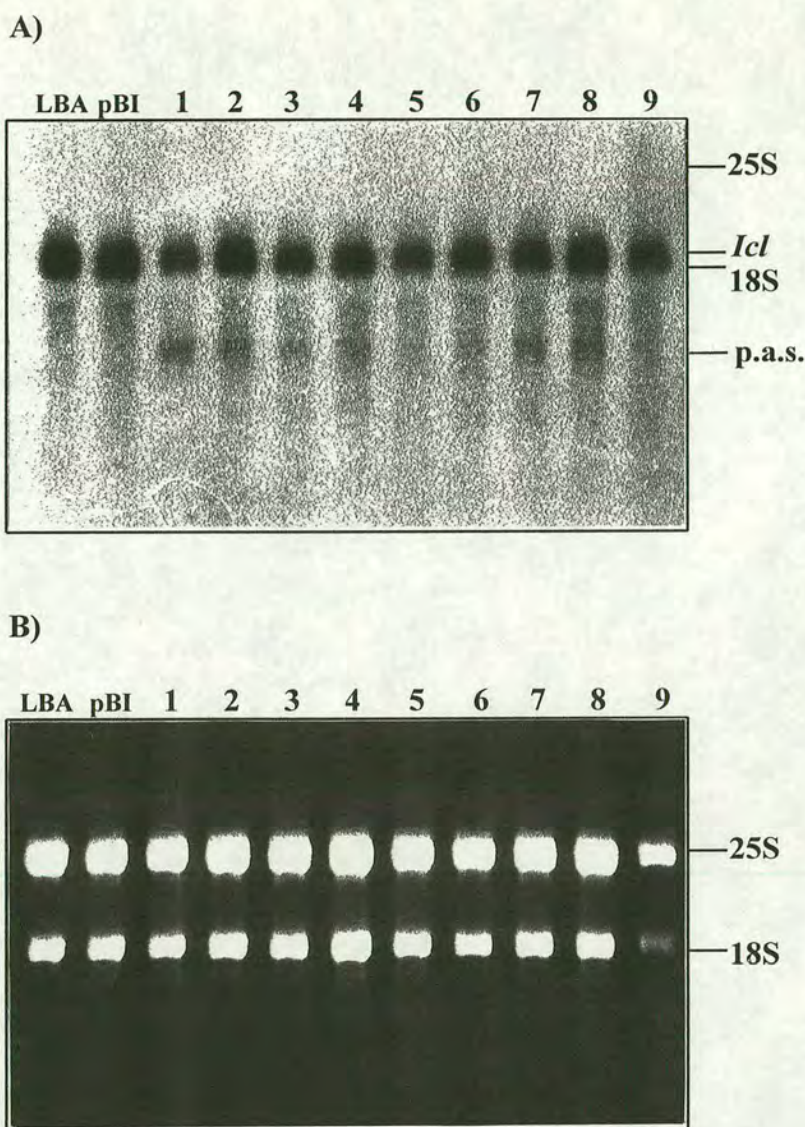




**Figure 4.17: TICL T<sub>1</sub> seedling screen, set 2.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5µg was loaded in each lane.

**A)** Northern blot hybridised with the *Icl* probe. **B)** Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. **Lane numbers** correspond to samples from the following TICL parent plants: **1:** 1.3. **2:** 7.2. **3:** 9.2. **4:** 21.1. **5:** 22.3. **6:** 25.1. **7:** 28.1. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. ***Icl*:** *Icl* mRNA. **p.a.s.:** putative antisense *Icl* RNA. **25S** and **18S:** positions of ribosomal RNA markers.





**Figure 4.18: TICL T<sub>1</sub> seedling screen, set 3.** Stage 3 seedlings were harvested and total RNA extracted, of which 2.5µg was loaded in each lane, except for TICL 25.2 (lane 9) for which less than 2.5 µg was available.

**A)** Northern blot hybridised with the *Icl* probe. **B)** Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. **Lane numbers** correspond to samples from the following TICL parent plants: **1:** 1.1. **2:** 7.1. **3:** 21.1. **4:** 12.1. **5:** 20.1. **6:** 20.2. **7:** 21.2. **8:** 24.1. **9:** 25.2. (lanes 1, 2 and 3 contain samples previously analysed in figures 4.16 and 4.17). **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. *Icl*: *Icl* mRNA. **p.a.s.:** putative antisense *Icl* RNA. **25S** and **18S:** positions of ribosomal RNA markers.



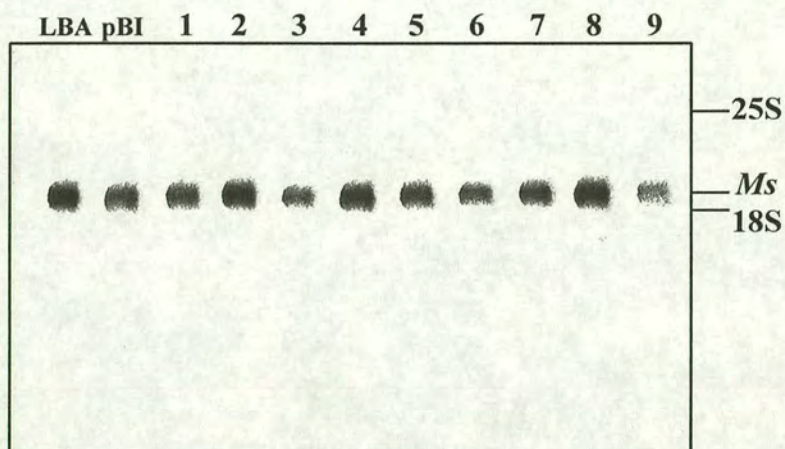
hybridised with the *Ms* probe. The result of this re-hybridisation is shown in figure 4.19. The pattern of slight differences between *Icl* mRNA signals that can be seen in figure 4.18a is reflected by those of the *Ms* mRNA in figure 4.19, suggesting that the differences observed in figure 4.18a were not specific to the *Icl* mRNA and therefore not clearly attributable to the presence of the transgene. However, in the samples which had been identified as having apparently lower *Icl* mRNA levels than controls in figure 4.18a, the differences are nevertheless more significant than those observed in figure 4.19, especially in the case of TICL 20.1. It was considered necessary to confirm this by analysis of T<sub>2</sub> seedlings, therefore seedlings from the batches which showed low *Icl* mRNA levels in figure 4.18a (TICL 1.1, 20.1, 21.1) and, in addition, TICL 7.1 and 12.1, were grown to maturity for propagation of the T<sub>2</sub> generation.

#### 4.6.4.2 Analysis of TICL T<sub>2</sub> seedlings

Twenty seedlings descended from the original TICL T<sub>0</sub> transformants TICL 1.1, 7.1, 12.1, 20.1 and 21.1 were chosen at random, grown to maturity and allowed to set seed, which was collected. The T<sub>1</sub> descendants of the five T<sub>0</sub> progenitors were named in the same way as T<sub>1</sub> descendants of the TMS T<sub>0</sub> transformants, resulting in five sets of twenty plants each which were named TICL 1.1.1 to 1.1.20, TICL 7.1.1 to 7.1.20, TICL 12.1.1. to 12.1.20, TICL 20.1.1 to 20.1.20 and TICL 21.1.1 to 21.1.20, respectively.

The T<sub>2</sub> seed collected from each of these plants was tested for kanamycin resistance. Only seed batches which contained exclusively kanamycin resistant seed, or batches which showed the highest kanamycin resistant : kanamycin sensitive seedling ratios available out of all twenty batches in a set, were selected for analysis by northern hybridisation. This was done in order to maximise the detectability of potential differences in *Icl* mRNA levels compared to controls. The T<sub>2</sub> seed batches selected were those originating from TICL 1.1.5, .6 and .7, TICL 7.1.1, .6, .9 and .13, TICL 12.1.1, .6, .15 and .16, TICL 20.1.5, .7, .12 and .17, and TICL 21.1.14, .16 and .18. Total RNA was extracted from stage 3 seedlings of each of these batches,



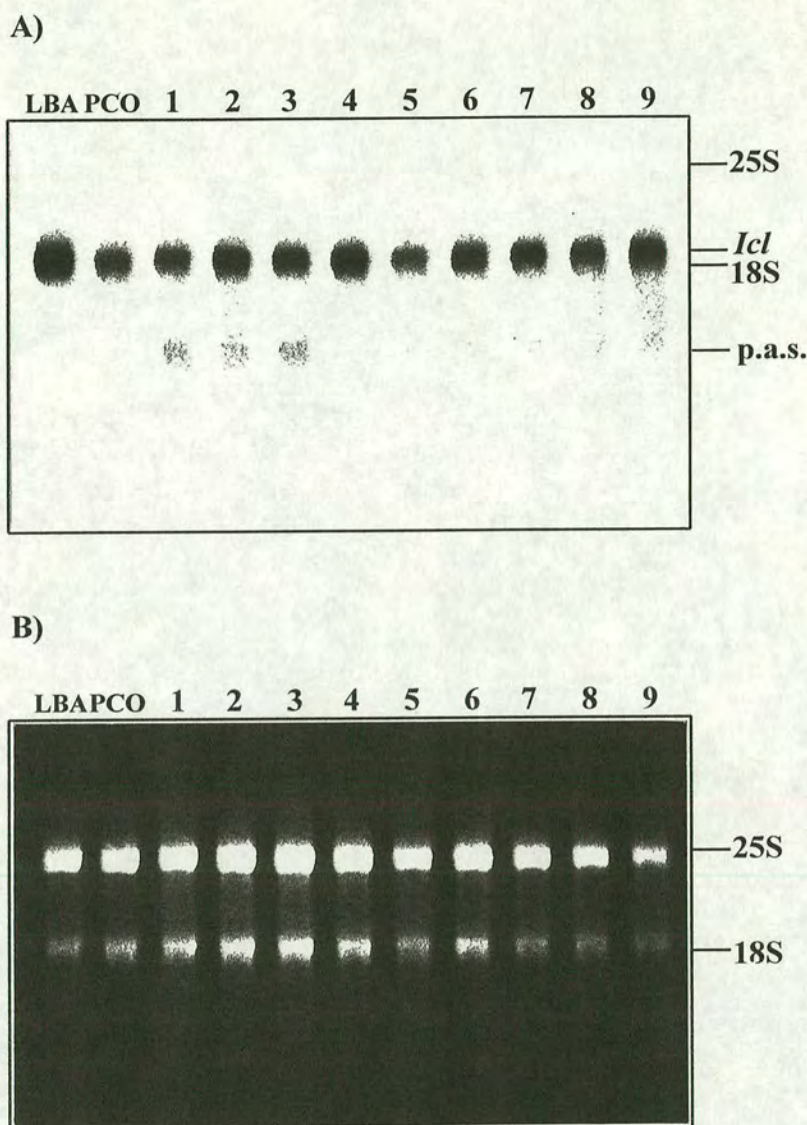


**Figure 4.19: TICL T<sub>1</sub> seedling screen, set 3, re-hybridised with the *N. plumbaginifolia* *Ms* probe.** The same blot as shown in figure 4.18, following removal of the *Icl* probe and re-hybridisation with the *Ms* probe. **Lane numbers** correspond to samples from the following TICL parent plants (as in figure 4.18): **1:** 1.1. **2:** 7.1. **3:** 21.1. **4:** 12.1. **5:** 20.1. **6:** 20.2. **7:** 21.2. **8:** 24.1. **9:** 25.2. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **pBI:** seedlings from a control plant carrying T-DNA from the pBI121 plasmid. ***Ms*:** *Ms* mRNA. **25S** and **18S:** positions of ribosomal RNA markers.



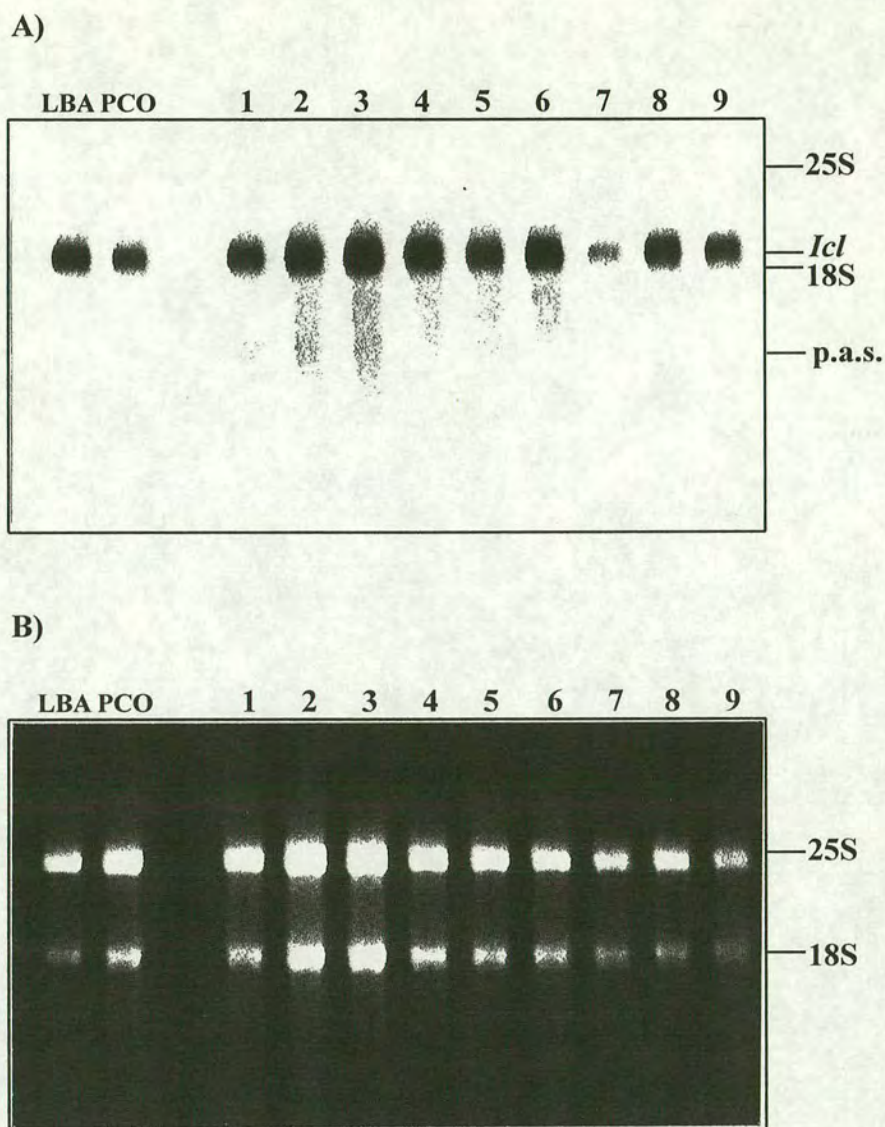
electrophoresed, transferred to nylon membrane and hybridised with the *Icl* probe. The result of this experiment (two northern blots) is shown in figure 4.20a and 4.21a, with the corresponding agarose gels depicted in figures 4.20b and 4.21b, respectively. In figure 4.20a, none of the TICL samples appear to have lower levels of *Icl* mRNA than the LBA4404 control. In figure 4.21a, however, one sample appeared to have a significantly lower level of *Icl* mRNA, TICL 21.1.14, compared to the LBA4404 control and other TICL samples. From fluorescence of ribosomal RNA on the corresponding agarose gel (figure 4.21b), this difference was clearly not attributable to reduced loading of total RNA. One other sample also appeared to have slightly lower levels of *Icl* mRNA, the control PCOR 1A stage 3 seedling RNA. However, the validity of this control was questioned in prior hybridisations (refer to section 4.6.3.3). Although co-suppression effects caused by the cucumber *Ms* promoter fragment in PCOR 1A plants (suggested in section 4.6.3.3) would have been unlikely to influence *Icl* mRNA steady-state levels in the way observed in figure 4.21a, the levels of *Ms* and *Icl* mRNA in all hybridisations with the PCOR 1A control appeared lower than in LBA4404 control samples, whilst never being the case with the pBI121 control. All seed batches analysed were obtained under the same growth conditions except the PCOR 1A seed batch, which had been obtained several years prior to all other batches. Taken together, these factors suggest that the PCOR 1A sample was less reliable as a control than the LBA4404 control. However, in order to establish whether or not the difference in *Icl* steady-state level in the TICL 21.1.14 sample was unequivocally attributable to the presence of the antisense *Icl* transgene and simultaneously confirm that the difference observed in the PCOR 1A sample was not exclusive to the *Icl* mRNA, the *Icl* probe was removed from the blot depicted in figure 4.21a and the blot re-hybridised with the *Ms* probe. The result of this re-hybridisation is shown in figure 4.22. As can be seen, the signal corresponding to the *Ms* mRNA in the PCOR 1A sample was as low as when the blot was hybridised with the *Icl* probe, showing that the accumulation of both *Icl* and *Ms* transcripts was lower in this control compared to the LBA4404 control. However, this was also the case with the TICL 21.1.14 sample. The *Ms* mRNA steady-state level was clearly as low in this sample as the *Icl* mRNA steady-state level, showing





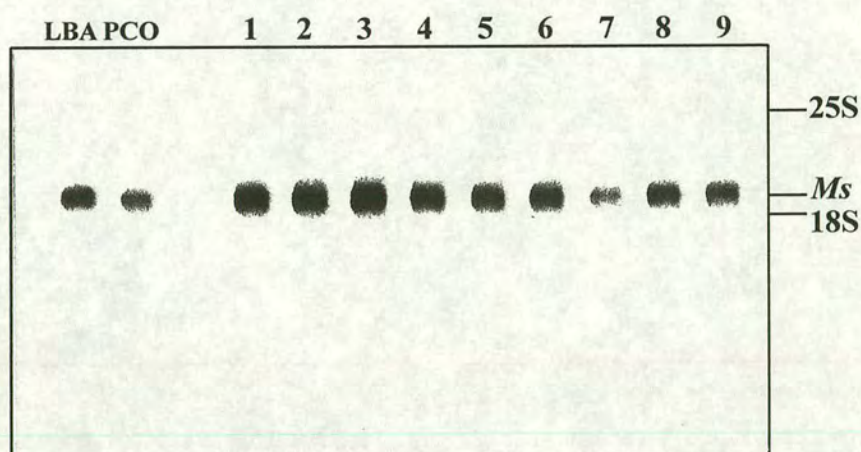
**Figure 4.20: Northern analysis of TICL  $T_2$  seedlings, set 1.** Stage 3 seedlings were harvested and total RNA extracted, of which 1.25  $\mu$ g was loaded in each lane. **A)** Northern blot hybridised with the *Icl* probe. **B)** Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. **Lane numbers** correspond to samples from the following  $T_1$  TICL parent plants: **1:** 1.1.5. **2:** 1.1.6. **3:** 1.1.7. **4:** 7.1.1. **5:** 7.1.6. **6:** 7.1.9. **7:** 7.1.13. **8:** 12.1.1. **9:** 12.1.6. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **PCO:** control sample from seedlings carrying T-DNA from the pBIPCOR plasmid. *Icl*: *Icl* mRNA. **p.a.s.:** putative antisense *Icl* RNA. **25S** and **18S:** positions of ribosomal RNA markers.





**Figure 4.21: Northern analysis of TICL T<sub>2</sub> seedlings, set 2.** Stage 3 seedlings were harvested and total RNA extracted, of which 1.25 µg was loaded in each lane. **A)** Northern blot hybridised with the *Icl* probe. **B)** Ethidium bromide-stained RNA gel which gave rise to the blot shown in A. **Lane numbers** correspond to samples from the following T<sub>1</sub> TICL parent plants: **1:** 12.1.15. **2:** 12.1.16. **3:** 20.1.5. **4:** 20.1.7. **5:** 20.1.12. **6:** 20.1.17. **7:** 21.1.14. **8:** 21.1.16. **9:** 21.1.18. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **PCO:** control sample from seedlings carrying T-DNA from the pBIPCOR plasmid. ***Icl*:** *Icl* mRNA. **p.a.s.:** putative antisense *Icl* RNA. **25S** and **18S:** positions of ribosomal RNA markers.





**Figure 4.22: TICL  $T_2$  seedling RNA, set 2, re-hybridised with the *N. plumbaginifolia* *Ms* probe.** The same blot as shown in figure 4.21, following removal of the *Icl* probe and re-hybridisation with the *Ms* probe. **Lane numbers** correspond to samples from the following  $T_1$  TICL parent plants (as in figure 4.21): **1:** 12.1.15. **2:** 12.1.16. **3:** 20.1.5. **4:** 20.1.7. **5:** 20.1.12. **6:** 20.1.17. **7:** 21.1.14. **8:** 21.1.16. **9:** 21.1.18. **LBA:** seedlings from a control plant regenerated from transformation with untransformed *A. tumefaciens* strain LBA4404. **PCO:** control sample from seedlings carrying T-DNA from the pBIPCOR plasmid. ***Ms*:** *Ms* mRNA. **25S** and **18S:** positions of ribosomal RNA markers.



that the difference in *Icl* steady-state level observed in figure 4.21a was not an effect exclusive to this mRNA. It was therefore not possible to unequivocally attribute the difference in *Icl* steady-state level in the TICL 21.1.14 sample to a putative effect of the antisense *Icl* transgene.

Inhibition of glyoxylate cycle enzyme synthesis may result in delayed germination or slower growth of seedlings following germination than seedlings with normal levels of these enzymes. The possibility therefore existed that selection of seedlings at a predetermined stage of post-germinative growth for northern analysis was effectively selecting against any putative slow-growing seedlings with inhibited *Ms* or *Icl* mRNA accumulation, by allowing them additional time, relative to faster-growing seedlings, to accumulate a critical level of *Ms* or *Icl* transcripts which may be necessary to reach stage 3 of post-germinative growth. Although no clear differences in the rate of post-germinative growth between transgenic and non-transgenic seedlings were ever noted, the natural variability in the timing of germination within and between seedling batches may have hidden any such effect. In order to establish whether there was any clear correlation between the steady-state level of *Icl* and the rates of either germination or post-germinative growth, these parameters were compared in seedlings from the LBA4404 and PCOR 1A controls and TICL 21.1.14 and 21.1.16. The LBA4404 and TICL 21.1.16 stage 3 RNA samples showed the same steady-state levels of *Icl*, which were taken as standard wild-type levels, whilst the PCOR 1A control and TICL 21.1.14 samples showed steady-state levels of *Icl* mRNA lower than these (refer to figure 4.21a). The rates of germination and post-germinative growth were compared by synchronising germination (as far as was possible) by imbibition of aliquots of at least 100 seeds from each of the four batches with GA<sub>3</sub> at 4 °C for an identical period of time for all batches (5 days), sowing all aliquots simultaneously, allowing germination under identical conditions and counting the number of seedlings which had reached stage 3 of post-germinative growth or beyond at 4 days post-imbibition. The LBA4404 seedling sample contained 117 seedlings at stage 3 or beyond, from a total of 277 seeds, 9 of which had failed to germinate. The PCOR 1A seeds had all failed to germinate by 4 days post-imbibition. The TICL 21.1.14 sample contained 145



seedlings at stage 3 or beyond, from a total of 179 seeds, 7 of which had failed to germinate. The TICL 21.1.16 sample contained 101 seedlings at stage 3 or beyond, from a total of 272 seeds, 92 of which had failed to germinate. The percentages of seedlings at stage 3 or beyond with respect to the total number sowed were therefore 42 % for LBA4404 (standard steady-state level of *Icl* mRNA), 0 % for PCOR 1A (lower steady-state level of *Icl* mRNA), 81 % for TICL 21.1.14 (lowest steady-state level of *Icl* mRNA) and 37 % for TICL 21.1.16 (normal steady-state level of *Icl* mRNA). Therefore there was no clear correlation between rates of germination or post-germinative growth and the steady-state levels of *Icl* mRNA as detected by northern analysis. The most notable datum above is that for the PCOR 1A sample, in which no seedlings had germinated at the time point selected. This supported the suggestion that unknown factors such as variations in growth conditions or age of seeds had considerable influence on the rate of germination or post-germinative growth and may also have been responsible for the fluctuations in *Ms* and *Icl* mRNA steady-state levels observed in the northern analysis of TMS and TICL seedlings.



## **5. DISCUSSION**



## 5.1 Isolation and characterisation of four new *Ms* and *Icl* cDNA fragments

### 5.1.1 PCR amplification of *Ms* and *Icl* cDNA fragments

The aim of this project was to generate transgenic plants containing chimaeric transgenes consisting of a fragment of endogenously-derived *Ms* or *Icl* cDNA in the antisense orientation with respect to the CaMV 35S promoter, with the intention of inhibiting synthesis of MS or ICL. The initial step was therefore the isolation of *Ms* and *Icl* cDNA fragments from the species with which these experiments were to be carried out, potato and *N. plumbaginifolia*, as neither the *Ms* nor the *Icl* cDNAs or genes had previously been isolated from these species. This was achieved by PCR amplification of cDNA fragments. This method was chosen for two reasons:

- i) it was deemed to be more rapid than construction and screening of cDNA libraries.
- ii) it was apparent from available nucleotide sequence information from other plant species that regions of the *Ms* and *Icl* cDNA sequences existed which were sufficiently highly conserved between species to design PCR primers with relatively low redundancy.

The most significant potential limitation of PCR was that it would not allow amplification of the complete coding region of either gene. This meant that a partial cDNA would have to be relied upon to cause antisense inhibition in the transgenic plants subsequently generated. Because of the choice of primers (refer to figure 2.3), the PCR fragments generated were to be amplified from an approximately central segment of the coding regions in all cases. Neither of these aspects were thought to be a significant limitation, as precedent studies in which antisense inhibition had been successfully achieved using partial cDNA fragments derived from different parts of the coding region were known to be extensive. These studies suggest that no single part of a coding region is consistently necessary to achieve antisense inhibition. Gene expression has been effectively inhibited with chimaeric antisense genes containing cDNA sequences from the 5' ends of coding regions (Smith *et al.*, 1988; Flachmann & Kühlbrandt, 1995; Rodermel *et al.*, 1988; Lee *et al.*, 1993;



Flipse *et al.*, 1996; Dwivedi *et al.*, 1994), sequences from central segments of coding regions (Sandler *et al.*, 1988; van der Krol *et al.*, 1990a) and sequences from the 3' ends of coding regions (van der Krol *et al.*, 1990a; Kuipers *et al.*, 1995). Although the widely postulated mechanism of antisense inhibition involving hybridisation of sense and antisense RNA (refer to section 1.6.3) implies that large antisense RNA molecules would have an inherent advantage over small antisense RNA molecules in causing inhibition (assuming both to be equally complementary to the target gene), this has been shown not to be consistently true. Studies involving comparisons of antisense cDNA fragments of different lengths have suggested that the significance of antisense RNA size is case-specific. Separate studies have shown large antisense fragments to be more effective (van der Krol *et al.*, 1990a), no more effective (Kuipers *et al.*, 1995) and less effective (Flipse *et al.*, 1996) than small fragments in causing antisense inhibition. The *Ms* and *Icl* cDNA fragments amplified for construction of the chimaeric antisense genes in this project included 45 % (*N. plumbaginifolia* and potato *Ms*) and 50 % (*N. plumbaginifolia* *Icl*) of the respective putative coding regions (42 % for potato *Icl*) in positions slightly 5' of centre with respect to the putative coding regions (refer to figure 2.3). In light of the inconsistent evidence for specific parts of coding regions being more effective than others in causing antisense inhibition, there was no reason to presuppose any intrinsic disadvantage in the cDNA fragments selected.

### **5.1.2 Cloning and characterisation of *Ms* and *Icl* cDNA fragments**

The PCR products obtained in amplification reactions were all of the sizes expected given the assumption that the relevant segments of the potato and *N. plumbaginifolia* *Ms* and *Icl* coding regions were the same sizes as those in the species analysed for design of PCR primers. It was concluded from partial nucleotide sequence analysis following cloning that the amplified products were the expected *Ms* and *Icl* cDNA fragments. The 5' and 3' ends of the cloned fragments showed high levels of nucleotide sequence identity with the relevant segments of *Ms* and *Icl* cDNAs from other organisms (the percentage identities for the 5' segments are listed in table 4.1),



the lowest levels of identity being consistently with *E. coli* and the highest with other higher plant species. In the case of the *Ms* cDNA sequence comparisons, the highest level of identity was obtained when potato and *N. plumbaginifolia* were compared with each other (87.5 %). This was not unexpected, as potato and *N. plumbaginifolia* were the only two members of the Solanaceae in the set of plant sequences compared (the potato and *N. plumbaginifolia* *Icl* cDNA fragment sequences could not be compared, as the sets of primers used for each were not the same and the cDNA fragments subsequently amplified were non-overlapping). Therefore, *Ms* and *Icl* cDNA fragments from two species for which these were previously unavailable were successfully cloned by the method described. The *N. plumbaginifolia* *Ms* cDNA fragment was also found to contain the hexanucleotide recognition site for the restriction endonuclease *Hind*III between nucleotides 144 and 145 from the 5' extremity of the cloned fragment.

The nucleotide sequences of the four cloned cDNA fragments were not fully determined, as time limitations dictated that plant transformations were an immediate priority following isolation of the cDNA fragments. The complete sequences of the fragments will be determined prior to any further use.

### **5.1.3 Analysis of *Ms* mRNA accumulation during post-germinative growth of *N. plumbaginifolia* seedlings**

The *N. plumbaginifolia* *Ms* cDNA fragment was used to synthesise a probe for northern analysis of wild-type seedlings and, subsequently, transgenic seedlings. Initially, the pattern of accumulation of *Ms* mRNA during post-germinative growth in wild-type seedlings was monitored using this probe. The hybridisation pattern of this probe with a northern blot of RNA extracted from seedlings at stages 0 to 7 inclusive (refer to figure 4.3) was consistent with the probe being genuine *Ms* cDNA, which hybridised to a single band of a size consistent with the band being *Ms* mRNA. It was concluded that this band was the *Ms* mRNA. The intensity of the *Ms* mRNA band changed greatly during the stages of post-germinative growth



monitored. The *Ms* mRNA was detectable in imbibed seed (stage 0), less so at stages 1 and 2, most abundant at stages 3 and 4, faintly detectable at stage 5 and undetectable by stages 6 and 7. These findings approximately correspond to previous data concerning the expression of the *Ms* gene in other plants during post-germinative growth (see section 1.4.3). However, in contrast to previous reports of the expression of the cucumber *Ms* gene at stage 0 (seed immediately after imbibition, Smith & Leaver, 1986; Weir *et al.*, 1980; Sarah *et al.*, 1996), the *Ms* transcript is clearly detectable at stage 0 in *N. plumbaginifolia* and in fact more abundant than in the following two stages. Assuming antisense RNA to be more effective in reducing levels of endogenous mRNA when in stoichiometric excess, this may have contributed to the difficulties encountered in finding TMS transformant seedling batches in which the antisense RNA had caused significantly low steady-state levels of endogenous *Ms* mRNA, as the *Ms* gene was clearly not inactive even in the period immediately following imbibition.

## 5.2 Analysis of transgenic plants

### 5.2.1 Transgenic *N. plumbaginifolia* seedlings undergo germination and post-germinative growth

Based on the assumption that the *nptII* gene and the CaMV 35S promoter-antisense cDNA fusion had remained linked in kanamycin resistant seedlings, it was concluded that the presence of the transgene did not in itself prevent the germination or subsequent growth of *N. plumbaginifolia*. Of the 59 T<sub>0</sub> TMS regenerants from the TMS transformation, 58 plants produced seed of which at least a proportion was kanamycin resistant and this was the case for all 23 T<sub>0</sub> regenerants from the TICL transformation. Plants carrying the transgene were therefore capable of sexual reproduction, contrary to the hypothesis which led to the transformation of potato in parallel. The potato transformations had been carried out in anticipation of sterility in some critical *N. plumbaginifolia* plants carrying the antisense *Ms* or *Icl* transgene. As this was found not to be the case, all further study involved only the *N.*



*plumbaginifolia* transformants. Even assuming co-segregation of the *nptII* gene and CaMV 35S promoter-antisense *Ms* cDNA fusion, the possibility nevertheless existed that the CaMV 35S promoter-antisense cDNA fusion was not expressed in seedlings which did express the *nptII* gene. However, the expression of the CaMV 35S promoter-antisense cDNA fusion was demonstrated in a large proportion of TMS plants, in which the antisense *Ms* RNA was shown to be efficiently synthesised. Co-inheritance of the *nptII* gene and the CaMV 35S promoter-antisense *Ms* cDNA fusion was supported by the absence of antisense *Ms* RNA exclusively from anther tissue from TMS B.33.1.5, whilst all other TMS plants whose anther tissue was tested showed high levels of antisense RNA (figure 4.11). Subsequently, TMS B.33.1.5 was found to be the only TMS plant whose anther tissue was analysed which had not been carrying the kanamycin resistance marker. Antisense RNA was also absent in seedling RNA from TMS A.6.1, the only TMS T<sub>0</sub> transformant to produce exclusively kanamycin sensitive T<sub>1</sub> progeny (refer to table 4.3 and figure 4.5). Although the identity of the putative *Icl* antisense RNA band was not confirmed by riboprobe experiments as had the antisense *Ms* RNA, it was also detectable (though more faintly) in a number of TICL seedling samples. Therefore, neither the presence alone nor the expression of the antisense transgene, as shown by the accumulation of antisense RNA, were sufficient to prevent either germination or post-germinative growth.

### ***5.2.2 A phenotype of reduced fertility observed in TMS and TICL plants was not caused by the presence of the transgene***

A significant proportion of TMS and TICL transformants displayed the LSP phenotype (refer to section 4.4.1), whereby seed pod development was severely impaired and seed production greatly reduced. Individual flowers on LSP plants could be rescued from this impairment by cross-pollination with wild-type pollen and thus the LSP phenotype must have been caused (at least in part) by the production of defective pollen by the anthers of LSP plants. This phenotype was consistent with the hypothesised effects of antisense inhibition of glyoxylate cycle function. However,



the T<sub>2</sub> progeny from a significant proportion of T<sub>1</sub> plants (derived from the TMS transformations) exhibiting the LSP phenotype were found not to have inherited the kanamycin resistance marker. Although it was possible that the *nptII* gene could have been segregated from the CaMV 35S promoter-antisense cDNA fusion in this generation, this would have been highly unlikely to occur in the number of cases observed. Alternatively, recombination between the *nptII* gene and the antisense transgene could have occurred during self-fertilisation of the T<sub>0</sub> TMS B.37.1 parent transformant, but the same event would have had to occur in the independent transformant TMS B.33.1 or its T<sub>1</sub> descendant B.33.1.5. In addition, accumulation of antisense *Ms* RNA in TMS seedlings did not correlate with the LSP phenotype in their parent plant, and plants likely to be carrying more than one copy of the transgene, as suggested by kanamycin resistance ratios in their progeny, did not necessarily display the LSP phenotype to any greater extent than plants apparently carrying fewer copies. It was concluded therefore that it was highly unlikely that the LSP phenotype had been caused by any effect of the antisense transgene.

### 5.2.3 Screening methodology

Analysis of plants transformed with the antisense *Ms* cDNA construct was initially carried out by assaying MS enzyme activity in crude protein extracts from seedlings. The stage of post-germinative growth in seedlings had a critical influence over the steady-state levels of *Ms* mRNA (discussed in section 5.1.3) and hence almost certainly also on the corresponding levels of enzyme activity, which was known to be the case in other species (discussed in section 1.4.3). It was therefore of great importance to standardise the stage of post-germinative growth in seedlings selected for analysis in order to account for this factor. Because of wide variation observed in the timing of germination even following the period of imbibition which was intended to synchronise germination, harvesting of seedlings at a specific time point after imbibition was not sufficient to standardise the stages of post-germinative growth between samples. Seedlings were therefore manually selected when they had reached stage 3 of post-germinative growth, as defined in section 2.1.1.



Although the timing of germination displayed considerable variation, the proportions of seed which eventually germinated in all TMS and TICL batches were no lower than wild-type. However, the asynchrony of germination made it problematic to assess either the rate of germination or the rate of growth of seedlings after germination. Therefore, it was possible that seedlings with delayed germination or slower post-germinative growth, phenotypes which were hypothesised as being possible consequences of glyoxylate cycle inhibition, were not being identified prior to selection for analysis. Any such putative seedlings were thereby being allowed to reach stage 3 of post-germinative growth before selection. If seedlings require threshold levels of glyoxylate cycle enzymes before they can reach any given stage of post-germinative growth, and therefore all seedlings at any given stage necessarily contain the same amounts, stage-specific selection may have had the effect of inadvertently making any low MS activities or mRNA steady-state levels undetectable. This possibility was subsequently discounted by accurate measurement of germination and growth rates in seedling batches which were known to have low steady-state levels of *Icl* mRNA (refer to section 4.6.4.2), whereby low steady-state levels were shown not to necessarily lead to delayed germination or slower post-germinative growth.

Despite standardisation of post-germinative growth stages, the MS assays displayed considerable variability, both between repeated assay readings for the same sample and the quality of the crude protein extracts obtained for each batch of seedlings. An alternative method of screening for plants in which the antisense transgene had been effective, was therefore necessary. Because a likely outcome of antisense inhibition was to cause MS or ICL protein levels to be lower than wild-type, the ideal screen would have been the testing of immunologically detectable MS or ICL in total seedling protein which had undergone denaturing polyacrylamide electrophoresis and transfer to nitrocellulose membrane (western blotting). Antibodies raised to *N. plumbaginifolia* MS and ICL were not available. Rather than test antibodies raised to MS and ICL from other species for their ability to recognise the *N. plumbaginifolia* proteins, a different approach was adopted. The method selected was analysis of mRNA steady-state levels by northern blotting. This method



was considered to be valid because antisense inhibition has been widely reported as manifesting itself as an inhibition of the accumulation of target gene mRNA (van der Krol *et al.*, 1988; Smith *et al.*, 1988; van der Krol *et al.*, 1990a; Flachmann & Kühlbrandt, 1995; Kuipers *et al.*, 1995; Robert *et al.*, 1989; Stockhaus *et al.*, 1990). Although a reduction in target gene mRNA steady-state levels does not always lead to a decrease in enzyme levels (Flachmann & Kühlbrandt, 1995), analysis of mRNA steady-state levels was considered to be the most rapid, reliable and informative approach.

#### **5.2.4 Northern analysis of TMS and TICL transformants**

In the same way as for the MS enzyme assays, stage 3 T<sub>1</sub> seedlings from all TMS and TICL transformants were manually selected for total RNA extraction and northern analysis in order to screen for low steady-state levels of *Ms* or *Icl* mRNA. None of the samples analysed showed significantly lower levels than controls, although a minor proportion showed small differences. The magnitude of the differences observed was such that equal loading of total RNA was critical in order to standardise the signals obtained. Spectrophotometric methods were used to initially determine loadings and these were confirmed or adjusted according to the fluorescence of ribosomal RNA bands under UV light. That this was a valid method of determining total RNA loading was confirmed by the fact that calmodulin mRNA, whose accumulation was unlikely to be affected by the manipulation of that of the *Ms* or *Icl* mRNA, was found to display a pattern of hybridisation which reflected exactly the fluorescence of ribosomal RNA.

Because the differences in *Ms* and *Icl* mRNA were minor, no firm conclusions were drawn from the results obtained from analysis of the T<sub>1</sub> generation with respect to the effectiveness of either antisense transgene. However, this initial screen indicated that they were failing to affect *Ms* or *Icl* transcript accumulation in stage 3 seedlings in any significant way. Although several T<sub>1</sub> seedling batches gave kanamycin resistance ratios significantly greater than that expected for normal



Mendelian inheritance of a single transgene insertion, all the T<sub>1</sub> seedling batches (except one completely kanamycin sensitive batch, TMS A.6.1) were mixtures of kanamycin resistant and kanamycin sensitive seedlings, the majority being mixtures of approximately 3 kanamycin resistant : 1 kanamycin sensitive. This would have had the effect of making any potential differences in *Ms* or *Icl* mRNA levels caused by either transgene apparently less significant than in batches containing exclusively transgenic seedlings. Transgenic lines displaying apparently low *Ms* or *Icl* mRNA steady-state levels relative to controls were therefore propagated to generate the T<sub>2</sub> generation. Because the T<sub>1</sub> generation was likely to contain plants homozygous for the transgene, the T<sub>2</sub> seed from such plants would be exclusively transgenic. However, with the exception of TICL 21.1.14 which is discussed below, even T<sub>2</sub> transformants which were apparently homozygous for the transgene failed to show mRNA levels significantly lower than those observed in the T<sub>1</sub> seedlings.

In the majority of TMS and TICL samples, a single band in addition to that which was identified as the mRNA, was detectable in northern blots. For the TMS samples, this additional band was shown to be antisense RNA complementary to the *Ms* cDNA fragment. The additional band in TMS and TICL samples was therefore a clear indication that the antisense transgenes were being correctly expressed. Within the TMS and TICL sample sets, the antisense *Ms* RNA and putative antisense *Icl* RNA bands varied widely in the intensities of hybridisation with the respective probes. This may have been due to the influence of sequences bordering the site of T-DNA insertion (position effects) in each independent transformant and is a phenomenon which has been widely reported (van der Krol *et al.*, 1988; Oliver *et al.*, 1993; Stockhaus *et al.*, 1990; Tieman *et al.*, 1992). On average, the antisense *Ms* RNA was more clearly detectable in TMS samples than the putative antisense *Icl* RNA was in the TICL samples. The putative antisense *Icl* RNA was significantly less abundant relative to the *Icl* mRNA than the antisense *Ms* RNA was to *Ms* mRNA. If this difference were solely due to effects related to the site of transgene insertion, it would have to imply a bias for insertion sites specific to each antisense construct, given that it was an effect common to independent transformants within each of the TMS and TICL sets of transformants.



The fact that the antisense *Ms* RNA (and putative *Icl* RNA) was visible in RNA samples was not unexpected as the detectability of antisense RNA has been reported in numerous studies (van der Krol *et al.*, 1988; Smith *et al.*, 1988; Oliver *et al.*, 1993; Flachmann & Kühlbrandt, 1995; Robert *et al.*, 1989; Stockhaus *et al.*, 1990; Tieman *et al.*, 1992, Sheehy *et al.*, 1988, Samac & Shah, 1994, Delauney *et al.*, 1988, Murfett *et al.*, 1995). This was not specific to seedling RNA, as the antisense *Ms* RNA was abundant in anther tissue also. In seedlings undergoing post-germinative growth, the steady-state levels of the antisense *Ms* and putative antisense *Icl* RNAs were never higher than their corresponding mRNAs, however. If the stoichiometric model of antisense inhibition is accepted, this may have been the main reason why the steady-state levels of neither the *Ms* nor the *Icl* mRNA were significantly lower than controls in TMS and TICL stage 3 seedling RNA. However, in an attempt to induce the expression of glyoxylate cycle genes by starvation, it was inadvertently discovered that kanamycin induces *Ms* gene expression in the presence of light. The reason for this effect was not investigated, but provided the desired circumstances under which the effect of an excess of antisense *Ms* RNA on *Ms* mRNA could be investigated. In this experimental system, an excess of antisense RNA did not reduce the steady-state level of *Ms* mRNA. Whether or not this was also the case in anther tissue could not be determined because whilst the antisense *Ms* RNA was abundant in this tissue, the *Ms* mRNA was not detectable. The abundance of antisense *Ms* RNA was unexpected, as it has been reported that the CaMV 35S promoter shows very low activity in anther tissue overall and is inactive in the tapetum and sporogenic cells (van der Meer *et al.*, 1992). Whether the antisense RNA was being synthesised in developing pollen as was intended, or cells other than those in which the glyoxylate cycle is thought to operate, could not be determined.

One TICL T<sub>2</sub> stage 3 seedling RNA sample was found to contain significantly low levels of *Icl* mRNA, TICL 21.1.14. This result was obtained in light of the fact that one of the controls, RNA extracted from homozygous stage 3 seedlings derived from transformation with the pBIPCOR plasmid, consistently showed slightly lower steady-state levels of both *Ms* and *Icl* mRNA than the LBA4404 control when analysed in northern blots. The result obtained for the PCOR 1A seedlings suggested



that the steady-state levels of glyoxylate cycle gene transcripts were affected by unidentified and, therefore, unaccounted-for factors. The most obvious differences between the PCOR 1A seedlings and the TMS and TICL seedlings was that the parent transformant had been generated and grown under different conditions and the seed was several years older than the TMS and TICL seed. Any or all of these factors may have had an influence on the expression of glyoxylate cycle genes. It was found that kanamycin causes strong induction of *Ms* gene expression in the presence of light (discussed above). This could be interpreted as a response to a physiological stress synergistically created by these two factors. The very fact that a previously unidentified set of conditions was inadvertently found to affect glyoxylate cycle gene expression suggests that the glyoxylate cycle may be open to influence from potentially numerous external factors. The response to kanamycin and light may be indicative of a general response to stress conditions. If this general response exists, then a wide variety of stress-inducing factors, such as poor growth conditions, might be expected to cause fluctuations in the expression of glyoxylate cycle genes and would be more likely to affect *Ms* and *Icl* simultaneously rather than either gene individually. The result obtained with the PCOR 1A control therefore put into question any low *Icl* mRNA steady-state levels in TICL samples which were reflected by concomitantly low steady-state levels of *Ms* mRNA, and the converse in TMS samples. This was in fact the case with the low *Icl* steady-state levels observed in TICL 21.1.14 by northern blotting. Removal of the *Icl* probe and re-hybridisation with the *Ms* probe showed that the transcripts of both genes were at similarly low steady-state levels.

Although this result suggested that the low *Icl* mRNA steady-state level observed in the seedlings of TICL 21.1.14 was not related specifically to the presence of the antisense *Icl* transgene, it did not prove this. In the absence of a detailed understanding of the coordination of expression of the *Ms* and *Icl* genes, it cannot be ruled out that the levels of expression of one gene might influence the expression of the other. The coordinated regulation of *Ms* and *Icl* gene expression at certain stages of development and under certain conditions (refer to section 1.4 and 1.5.2.2), and the possibility of a close enzymatic association (refer to section 1.2.5), suggest that



mechanisms of co-regulation might exist. Such putative mechanisms might result in the levels of gene expression of either *Ms* or *Icl* having influence over that of the other. The results with the PCOR 1A control seedlings would not necessarily be incompatible with this as the *Ms* promoter fragment in the pBIPCOR plasmid could conceivably have had co-suppression effects on *Ms* expression, in which case the regulatory mechanism postulated above might also apply. Therefore, although the results of northern analysis of TMS and TICL seedlings suggested that the differences in the steady-state levels of target gene transcripts were not attributable to the presence of the transgene, the possibility of a causal relationship could not be excluded.

Thus, in stage 3 seedlings from the majority of transformants, the antisense *Ms* and *Icl* transgenes did not cause detectable differences in the steady-state levels of target gene mRNA, whilst in the few transformants in which low steady-state levels compared to controls were observed, the differences could not be unequivocally attributed to the presence of the transgene. This was despite the detectable presence, in some cases abundance, of antisense *Ms* RNA in most TMS samples and putative antisense *Icl* RNA in most TICL samples. The question of why the successfully synthesised antisense RNA failed to affect levels of target gene mRNA has to be addressed. The possible reasons for this are discussed below.

## **5.2.5 Factors influencing antisense inhibition**

### **5.2.5.1 cDNA fragments used in chimaeric antisense gene construction**

The antisense RNA may have been ineffective due to the segment of cDNA selected for construction of the plasmids for plant transformation. There is no clear consensus concerning the most effective part of the coding region for inhibition. The cDNA fragments isolated in this study satisfied the documented criteria for effective inhibition in terms of the part of the coding region included and the overall length of the fragment used (discussed in sections 1.6.2.1 and 5.1.1). However, the case-



specific nature of antisense inhibition suggests that fulfilment of these criteria does not necessarily mean that the fragment selected will be effective. The use of full length cDNA clones may have increased the probability of successful inhibition of *Ms* or *Icl* expression, in light of the ineffectiveness of the partial cDNAs used.

#### 5.2.5.2 *Stoichiometry of antisense and sense RNA*

The amounts of antisense RNA required to cause inhibition may have needed to be higher than those of the target gene mRNA. This might have been the case if antisense inhibition in this system operated by pairing of antisense RNA with mRNA followed by simultaneous processing of both. Such a process would have required a stoichiometric excess of antisense RNA, which was known from northern analysis of post-germinative seedlings to have not been achieved in any samples. However, when kanamycin and light were found to induce expression of the *Ms* gene (and the antisense *Ms* transgene) in older seedlings, an excess of antisense RNA was found to have no detectable effect on the steady-state levels of the *Ms* mRNA. This suggests that unless seedlings undergoing post-germinative growth had been any more susceptible to the effects of antisense RNA than were the seedlings in this experiment, it is unlikely that an excess of antisense RNA would have detectably reduced the steady-state levels of *Ms* mRNA in stage 3 seedlings. In addition, evidence suggests that a stoichiometric excess of antisense RNA is not a prerequisite for effective inhibition (Stockhaus *et al.*, 1990; van der Krol *et al.*, 1990a; Cannon *et al.*, 1990; discussed in section 1.6.3). If a simple stoichiometric mechanism were applicable, it might have been expected that the steady-state levels of *Ms* or *Icl* mRNA would be lowest in samples in which the antisense RNA steady-state levels were highest, but this was not found to be the case. This was in contrast to studies in which a correlation between the degree of inhibition and the levels of antisense RNA was established (Robert *et al.*, 1989; Delauney *et al.*, 1988). One study has suggested that high levels of transgene expression can trigger self-silencing (Elmayan & Vaucheret, 1996). In this study, *N. tabacum* was transformed with a bacterial gene (in some cases a single copy) with no endogenous counterpart in the plant host, under



the control of the CaMV 35S promoter modified by addition of enhancers to increase levels of expression. Initial expression of the transgene early in development was post-transcriptionally silenced in mature plants. The degree of silencing in each transformant positively correlated with the initial level of transgene expression, suggesting that expression of the transgene above a threshold level was responsible for its subsequent silencing. Therefore, enhancement of the expression of the antisense *Ms* or *Icl* transgenes should not only have been unnecessary for inhibition, but could potentially have been disadvantageous to their own expression.

#### 5.2.5.3 Genomic site of T-DNA integration

A third consideration with respect to the mechanism of antisense is the genomic location of the T-DNA insertions. The most significant potential effect of this would be the influence exerted on transgene expression by the relative activity of the region of the genome into which the T-DNA inserted. However, in the majority of TMS and TICL samples, this factor was clearly not preventing expression of the transgene, as antisense RNA was known to be synthesised. Therefore, if genomic position had influenced the effectiveness of the antisense transgenes, it would have had to do so by means other than affecting expression. It has been suggested (Park *et al.*, 1996) that a telomeric location is advantageous for silencing loci as it might permit telomeric loci to search the genome for homology. The evidence supporting this suggestion was obtained from studies of co-suppression and implies a model of target gene inactivation different from the model of antisense inhibition involving interaction of sense and antisense RNA. The two systems are therefore not necessarily analogous.

#### 5.2.5.4 Choice of species

It is possible that the species of plant in which antisense experiments are carried out influences the outcome. Whilst *N. tabacum*, tomato and potato have been extensively



used in antisense experiments, the use of *N. plumbaginifolia* is not common (Bourque, 1995). However, successful inhibition in *N. plumbaginifolia* has been reported (Cannon *et al.*, 1990; Murfett *et al.*, 1995), suggesting that this species lacks none of the mechanisms necessary for antisense inhibition to take place. The wide variety of species in which inhibition has been successful (Bourque, 1995) demonstrates that such mechanisms are not restricted to any specific species.

#### 5.2.5.5 *Tissues expressing the transgenes*

A final explanation for the ineffectiveness of the antisense transgenes is that they were expressed in different tissues to the *Ms* and *Icl* genes. Expression of *Ms* and *Icl* in seedlings undergoing post-germinative growth is not found throughout all organs but is confined to certain tissues, primarily in the cotyledons (Graham *et al.*, 1990; Reynolds & Smith, 1995b). It is therefore possible that if the transgenes were expressed in tissues in which *Ms* or *Icl* are not, no effect on *Ms* or *Icl* gene expression would be observed in RNA extracted from whole seedlings. The extensive use of the CaMV 35S promoter for successfully directing expression of antisense transgenes (Bourque, 1995) would suggest that it is unlikely that this promoter is inactive in any major tissue types such as cotyledon tissue. However, the CaMV 35S promoter is not thought to be constitutively active in all tissue types, notable exceptions being the tapetal and sporogenic tissues of anthers (van der Meer *et al.*, 1992).

#### 5.2.5.6 *Factors applicable to both transgenes*

The considerations above must be assessed in light of the fact that the antisense *Ms* and antisense *Icl* transgenes behaved in the same way. With both transgenes, an accumulation of antisense RNA failed to inhibit accumulation of the respective target gene mRNAs, despite the fact that the sequences of the cDNA fragments used to construct the chimaeric antisense genes were completely different. In addition, genomic location is unlikely to have affected independent transformants, in both sets



derived from transformation with either antisense gene, in the same way with respect to effects on target gene mRNA. This could only have been the case if all T-DNA insertion events led to integration in a region of the genome where expression of the transgene was allowed but inhibition of the target gene somehow prevented in spite of this.

If one or other of these factors were responsible for the failure of the antisense genes, they would therefore have had to affect both antisense transgenes in the same way. The most likely explanation must involve features common to both transgenes. This includes the fact that both transgenes were constructed with segments of cDNA located in the central part of the putative coding regions, and that the antisense RNA (putative in TICL samples) in stage 3 seedling RNA samples from both sets of transformants was always in sub-stoichiometric amounts with respect to the target gene mRNA. Either of these two reasons could be responsible for the lack of effect on the steady-state levels of target gene mRNA, but in light of the result with kanamycin-induced *Ms* expression (discussed in section 5.2.5.2), the effectiveness of the cDNA fragments used in the constructs seems more likely to have been a significant factor.

Any putative disadvantages due to the choice of *N. plumbaginifolia* or spatial separation of the sites of antisense and sense RNA synthesis, due to tissue-specific confinement of transgene expression, would be expected to affect both transgenes in the same way. There is evidence to suggest that *N. plumbaginifolia* has the necessary cellular mechanisms for antisense inhibition (Cannon *et al.*, 1990; Murfett *et al.*, 1995). Of these two factors, spatial separation of the sites of sense and antisense RNA synthesis would therefore seem the most likely. Although there is no evidence to suggest that the CaMV 35S promoter is not active in the same tissues and cells as the *Ms* and *Icl* genes, this possibility cannot be excluded.

A factor not directly related to the mechanism of antisense inhibition which would apply to both transgenes, is potential selection against transformants in which *Ms* or *Icl* expression was effectively inhibited due to one or other of the experimental procedures employed. An experimental procedure which fell into this category was



stage-specific selection of seedlings, and was accounted for (refer to section 4.6.4.2). It is possible that transformants with effectively inhibited *Ms* or *Icl* expression were being selected against during regeneration from callus culture. This might have occurred if regeneration was in some way dependent on the glyoxylate cycle. Although there is no evidence to suggest that *Ms* or *Icl* are expressed in callus or regenerating shoots under the culture conditions employed, this possibility cannot be excluded. However, given the presence of sucrose in the transformation and regeneration media (refer to section 2.5.1) it would seem unlikely that the glyoxylate cycle is critical in transformant regeneration to the extent that transformants lacking in glyoxylate cycle function would specifically fail to be regenerated.



## **6. CONCLUSIONS AND FUTURE WORK**



## 6.1 Conclusions

Four new cDNA fragments of *Ms* and *Icl* from *N. plumbaginifolia* and potato have been cloned. These fragments were used to construct chimaeric antisense genes with which each species was transformed. A total of 59 *N. plumbaginifolia* plants were regenerated from transformation with the chimaeric gene containing the *N. plumbaginifolia* antisense *Ms* cDNA fragment and 23 from transformation with the chimaeric gene containing the antisense *N. plumbaginifolia* *Icl* cDNA fragment, whilst 15 potato plants were regenerated from transformation with the chimaeric gene containing the potato antisense *Ms* cDNA fragment and 19 from transformation with the chimaeric gene containing the potato antisense *Icl* cDNA fragment.

A large proportion of the *N. plumbaginifolia* transformants appeared to carry two or more unlinked copies of the respective transgene. A phenotype of reduced fertility was found not to be dependent on inheritance of the transgene. In stage 3 seedling<sup>S</sup>/total RNA samples from the majority of TMS transformants, antisense RNA which was thought to have originated from transcription of the transgene, was clearly detectable and in some cases abundant. Though less clearly detectable, this was also the case for putative antisense RNA in TICL samples. Steady-state levels of antisense RNA were lower than target gene mRNA steady-state levels in all cases.

In several T<sub>1</sub> seedling RNA samples, target gene mRNA steady-state levels were found to be slightly lower than in control samples. However, when the T<sub>2</sub> progeny of mature T<sub>1</sub> plants were generated and analysed, in only one case (TICL 21.1.14) did the target gene mRNA steady-state level appear significantly low. Following removal of the *Icl* probe from this northern blot and rehybridisation with the *Ms* probe, it was apparent that *Ms* steady-state levels were equally low in this sample. This put in doubt that the difference in *Icl* mRNA steady-state level was specifically attributable to the presence of the antisense *Icl* transgene. Results obtained from attempts to enhance the relative accumulation of antisense RNA, and with one of the control samples, PCOR 1A, suggested that as yet undefined factors influence glyoxylate cycle gene expression. These may have been responsible for the low *Icl* mRNA steady-state level observed in the RNA extracted from seedlings



descended from TICL 21.1.14. However, in the light of limited understanding of glyoxylate cycle gene control and the mechanisms of antisense inhibition, it could not be determined from the results obtained whether or not the low level observed was attributable to the presence of the transgene.

## 6.2 Future work

The *Ms* and *Icl* probes, synthesised using the *N. plumbaginifolia* cDNA fragments as templates, could be employed to screen a stage 3 seedling cDNA library in order to isolate the full length *N. plumbaginifolia* *Ms* and *Icl* cDNA clones. Aside from the intrinsic value of characterising the full length cDNAs, this would be productive for two purposes. The first of these would be the ability to synthesise the MS and ICL proteins, from translation of the full length cDNAs, to which polyclonal antibodies for use in western analysis could be raised (antibodies could also be raised to partial polypeptides but would not maximise the number of recognisable epitopes). Western analysis of protein extracts from stage 3 TMS and TICL seedlings would be a direct means of observing effects on protein levels in transformants and would extend the results obtained by northern analysis. Protein levels can be reduced by antisense inhibition even when mRNA levels have not been affected (Oliver *et al.*, 1993). The second purpose of obtaining full length cDNA clones would be the potential for regenerating a second set of *Ms* and *Icl* transformants carrying the full length cDNAs in the antisense orientation with respect to the CaMV 35S promoter, or a modified version of this. Sense orientation constructs could also be used in an attempt to cause co-suppression of the target genes. Use of the *Ms* or *Icl* promoters to direct transcription of the antisense cDNAs concomitantly with the endogenous genes would allow accumulation of stoichiometrically equal amounts of endogenous gene transcripts and transgene transcripts, during developmental stages when the endogenous genes are expressed. This would be particularly advantageous during post-germinative growth, when the antisense *Ms* transgene in this study appeared less active, or had delayed activity, with respect to the *Ms* gene (refer to figure 4.15). Although the failure of the partial cDNA fragments used in this study suggests



strongly that use of full length cDNAs would not guarantee effective inhibition, use of full length clones would maximise the chances of inhibition if the problems encountered in this study were due to limitations of fragment length or position with respect to the complete coding region.

Comparison of the spatial localisation of *Ms* and *Icl* expression (Graham *et al.*, 1990; Reynolds & Smith, 1995b) with that of the CaMV 35S promoter (by transformation of *N. plumbaginifolia* with the CaMV 35S promoter-*gus* fusion) would determine whether they coincide or are confined to partially or non-overlapping tissues. Spatial separation would be significant with respect to the effectiveness of the antisense transgenes in this study, as cellular boundaries between sense and antisense RNA would probably preclude inhibition. This would certainly be the case if the mechanism of antisense inhibition is assumed to involve hybridisation of target gene mRNA with antisense RNA (refer to section 1.6.3). As with difficulties caused by temporally non-overlapping expression of the transgenes and target genes (discussed above), use of the target gene promoters to direct antisense transgene transcription would ensure spatially overlapping expression of transgenes and endogenous genes.

Induction of *Ms* and *Icl* expression, to the extent required, by starvation was not successful in this study, whilst the induction by the combination of kanamycin and light may have had undetected pleiotropic effects not associated with starvation. Optimisation of starvation treatments (for either TMS and TICL transformants already generated or transformed with antisense full length *Ms* or *Icl* cDNAs) would allow analysis of the effects of the antisense transgenes under physiologically relevant conditions in which they are not at a disadvantage with respect to the temporal expression of the target gene, as discussed in section 4.6.2.

Although *N. plumbaginifolia* has a relatively short generation time and was an amenable species for transformation and analysis, by these criteria *A. thaliana* would be the most appropriate choice for generation of future sets of antisense *Ms* or *Icl* transformants. However, the same potential problems of infertility due to effective inhibition of MS or ICL synthesis would apply to this species. The potato antisense



*Ms* and *Icl* (PMS and PICL) transformants generated in the early part of this project still provide an opportunity to screen for transformants in which the antisense transgenes have been effective, as the results obtained with the TMS and TICL transformants do not necessarily exclude the possibility that the transgenes have been more effective in the potato transformants. In the case of potential generation of antisense *Ms* or *Icl* transformants in *A. thaliana*, a set of potato transformants would be generated in parallel to account for the possibility of infertility caused by effective inhibition, as was hypothesised for the *N. plumbaginifolia* transformants.

An alternative method of generating plants in which MS or ICL synthesis has been inhibited or abolished is through T-DNA insertional mutagenesis in *A. thaliana* (Krysan *et al.*, 1996; McKinney *et al.*, 1995). Mutants in which the *Ms* or *Icl* genes have been disrupted by insertion of T-DNA could be screened in a heterozygous state, circumventing the potential problem of lethality in null mutants (Krysan *et al.*, 1996). If lethal during certain stages of development (for example embryo development or post-germinative growth) but not others (for example senescence), the effects of homozygous null *Ms* or *Icl* mutants during non-lethal stages of development could be studied by co-transformation with an *Ms* or *Icl* construct under the control of promoters specifically induced during the critical stage of development (for example a germination or embryogenesis-specific promoter). This would allow survival of mutants beyond stages of development when lack of MS or ICL would otherwise be lethal. Alternatively, supplying a utilisable carbon source during critical stages of development may serve the same purpose and would be particularly appropriate during post-germinative growth, for which a utilisable carbon source could be added to the germination medium. A converse approach could involve co-transformation with *Ms* or *Icl* cDNAs under the control of their normal respective promoters in which the control element corresponding to the stage of development which is under investigation has been deleted (Reynolds & Smith, 1995b; Sarah *et al.*, 1996). This would result in plants deficient in *Ms* or *Icl* expression during a specific chosen stage of development. Such a scheme would require either the use of promoter constructs from species for which they are presently available, or isolation and characterisation of the promoters of the *A. thaliana* *Ms* or *Icl* genes.



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